Lung Cancer
Strategies for Diagnosis and Treatment

Edited by Alba Fabiola Costa Torres

LUNG CANCER - STRATEGIES FOR DIAGNOSIS AND TREATMENT

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Meet the editor

Dr. Alba Fabiola Costa Torres has a Bachelor's degree in Pharmaceutical Sciences (2005) and an Improvement Course in Industrial Pharmacy (2007) from the Federal University of Paraiba, UFPB, Joao Pessoa, Brazil; a Master's degree in Pharmaceutical Sciences (2009) and a PhD in Development and Technological Innovation of Medicines (2013) from the Federal University of Ceara, UFC, Fortaleza, Brazil; and a Post-Doctoral fellowship in Pathology/Oncology from Johns Hopkins Medical School, JHMI, Baltimore, United States (2017). During the last few years the development of knowledge and technical/scientific experience in surgical pathology/oncology has been a major field of work, with urological and thoracic pathology being the main subarea of interest.

Contents

Preface

Over the last 100 years, lung cancer has been on the rise in the field of oncology, due to the challenges involved both in its causal factors and in diagnosis and treatment. In the distant past it was almost unreported and considered a very rare manifestation, but currently it steals the scene in the number of new cases recorded annually. In an inquiring oncology world scenario, where practical research efforts increasingly aim to improve human health, quality of life, and lifestyle, early and precise diagnosis and effective treatment methods able to guarantee a cure are the goal.

Obviously, a deeper understanding of how lung cancer develops and its genomic hallmarks are the watershed in the clinical management of these cases. In this respect, the improvement in imaging techniques to detect lung cancer and also an understanding of the molecular biology factors involved in lung tumor development and behavior offer a window of opportunities to research development in this area. Throughout the last few years there have been significant advances in imaging and surgical techniques, histological classification, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Also, although not applicable in clinical practice, lung cancer screening is being extensively studied and discussed, because early diagnosis is determinant to the progress of the disease. All these points compose the antitumor strategies in the battlefield against lung cancer.

This book presents to the reader selected examples of strategies on lung cancer diagnosis and treatment. With this aim the book is divided into two sections, where the first explores the diagnosis field and the second explores the treatment field. I hope that readers enjoy the book and that it contributes to improving the knowledge of and gives insights into better practices aimed at helping lung cancer patients reach a better quality of life and/or cure.

To the authors, I am very thankful for sharing their knowledge and contributions to this work, which can only be the result of constant and hard work. Also, I want to manifest my gratitude to the Intech editorial office for all their support, which made this book possible.

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Insights on Diagnosis of Lung Cancer

Precise Diagnosis of Histological Type of Lung Carcinoma: The First Step in Personalized Therapy

Jelena Stojšić

Additional information is available at the end of the chapter

Abstract

This chapter is a combination of personal experience of a pulmonary pathologist and available references in the diagnosis of non-small cell lung cancer (NSCLC) types. The morphological appearance of poorly differentiated lung carcinoma is not characteristic, so immunohistochemical staining is used for further differentiation. In order to save tumor tissue from paraffin blocks, the most rational way is to use only two antibodies, p40 for squamous cell carcinoma and TTF-1 for adenocarcinoma of the lung, and if necessary or if cancer growth is organoid, also one of two neuroendocrine markers (CD56 or Synaptophysin) can be used. If there is enough tumor tissue in the paraffin block to confirm the diagnosis, NapsinA, p63, Cytokeratin5/6 or Cytokeratin5 can be used. It should be kept in mind that no antibody is highly specific for one histological type of carcinoma or its origin and if the immunohistochemical finding is unspecific, it should be concluded that this is "not otherwise specified" (NOS) carcinoma. The rest of tissue must be preserved for current and future molecular testing and predictive immunohistochemical staining for the purpose of personalized NSCLC therapy.

Keywords: non-small cell lung cancer, diagnosis, immunohistochemical staining, paraffin block, TTF-1, p40, p63, CD56, Synaptophysin, NapsinA, Cytokeratin5/6, Cytokeratin5

1. Introduction

1.1. Epidemiology as basis for developing a strategy in the treatment of advanced non-small cell lung cancer

Lung carcinoma is the most commonly diagnosed malignancy worldwide. There is a different lung cancer incidence and mortality statistics throughout the world. In male population, lung

cancer has the highest incidence rate, especially in developing countries, while in developed countries, it is immediately behind the prostate cancer. In female population, the incidence rate of lung cancer is rising, and it is higher than cervical carcinoma, but still lower than breast cancer. Lung carcinoma mortality rate is still alarmingly high, both in developing and developed countries [1–3].

This high mortality rate has led to research of drugs, which will, in the era of personalized therapy, prolong the survival of diseased patients for more than 5 years and also improve the quality of their lives during and after the treatment. Individual approach to the treatment of lung cancer is based on a precisely diagnosed pathohistological type of non-small cell lung cancer (NSCLC) [4, 5].

1.2. Types of biopsy specimens and pathohistological classification of lung cancer

Two most common histological types of NSCLC are adenocarcinoma and squamocellular carcinoma [6–8]. At the time of diagnosis, about 75% of lung cancer is in an inoperable, advanced stage and less than 15% of patients survive more than 5 years. As these patients cannot be surgically treated, diagnosis of NSCLC is based on bronchoscopic and fine-needle aspiration biopsy (*fnab*) or video-assisted thoracic surgery (*VATS*). That is why there is a huge responsibility on the shoulders of the pathologist to diagnose a histological type of NSCLC on a small biopsy specimen and preserve paraffin-embedded carcinoma tissue for further genetic and immunohistochemical testing in order to determine the effective personalized therapy. It aims at prolonging survival of patients and improving the quality of their lives during and after the therapy [9, 10].

In the final interpretation of the pathohistological findings, pathologists use the classification of the World Health Organization (WHO) from 2004 [6], that is, an improved version from 2015 [7].

According to WHO classification from 2004, histological subtypes of NSCLC are as follows:

- squamous cell carcinoma (**Figure 1**)
- adenocarcinoma (**Figure 2**)
- large-cell carcinoma
- adenosquamous carcinoma
- sarcomatoid carcinoma
- carcinoid tumors
- salivary gland tumors.

Each of these histological NSCLC subtypes has its own variants that are diagnosed based on their morphological picture and specific immunophenotype [6].

In the WHO classification of lung carcinoma from 2015, there have been some changes because adenocarcinoma took over the first place from squamous cell carcinoma, which previously had primacy. The greatest change in this classification compared to the previous one
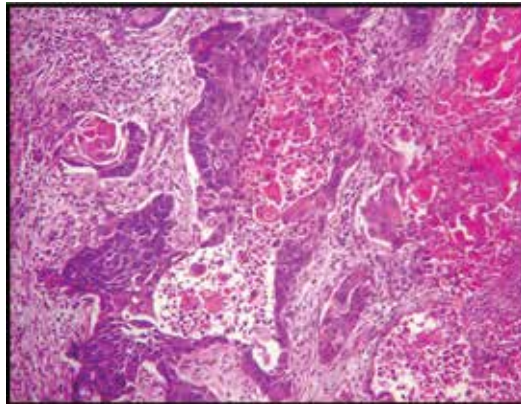
Figure 1. Keratinizing squamous cell carcinoma of the lung, H&E 100×.
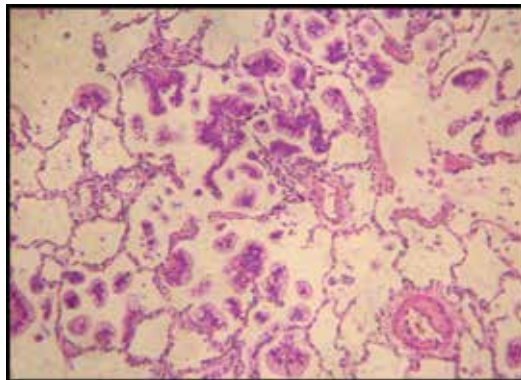
Figure 2. Adenocarcinoma of the lung with lepidic growth pattern, H&E 100×.

is a grouping of all carcinoma with neuroendocrine differentiation: carcinoid tumors, typical carcinomas (TC) and atypical carcinomas (AC), large-cell neuroendocrine carcinomas (LCC-NEC) and small-cell neuroendocrine carcinomas (SCLC) into one group of carcinomas due to specific biological behavior and special therapy, regardless of the different morphological picture. Remaining classification is identical to that of 2004 [7].

1.3. Processing of lung carcinoma tissue samples taken during bronchoscopy, FNA biopsy or VATS method

Bioptized lung samples obtained during bronchoscopy are fixed in 10% buffered formalin and brought to the laboratory. It is considered that sampling of tissue is representative if at least five biopsy samples have been delivered in diameter larger than 2 mm. Tissue samples taken during FNA biopsy should be delivered in 10% buffered formalin in the form of punc-tuate cylinder in order to use the whole tissue material for which is believed to contain lung cancer for morphological analysis and for immunohistochemical staining, while the rest of the tissue would be preserved for molecular testing (EGFR, optional KRAS), predictive

immunohistochemical staining (ALK, ROS1 and PDL-1), as well as for fluorescent *in situ* hybridization (FISH) if this method is accepted by consensus [11].

Basic information about the patient which is to be submitted in the biopsy referral for the pathohistological laboratory is: name and surname, gender, age, place of residence, occupation and smoking status. It is necessary to deliver clear and concise clinical picture, for example, whether there is a suspect tumor shadow in the lung, mediastinal lymphadenopathy, superior vena cava syndrome (SVCS), and so on. Also, it is necessary to note that there is a previously diagnosed pulmonary or some other kind of malignancy in patient, which histological type of tumor is diagnosed on that occasion and how long before the current examination. This data help to set a new pathohistological diagnosis by applying a smaller number of immunohisto-chemical staining to prove metastatic malignancy or a new primary carcinoma of the lung. An endoscopic finding of the mode of tumor growth and its localization must also be given. In this way, we save tumor tissue for methods which would be used in personalized therapy for advanced lung cancer. If these data are not available, the pathologist should consider that this is a biopsy of primary lung cancer. Incomplete data because of sloppiness and lack of interest of the doctor who performed the biopsy can lead to vagueness and difficulty in diagnosing and thus, to disrespect of the patient and pathologist. Correct communication at the patient-clinician level and clinician-pathologist level is the basis for setting a precise diagnosis.

According to NSCLC morphology, many pathologists would not agree on a definitive diag-nosis, but after immunohistochemical stainings, the same diagnosis should be made in a high percentage of them. Also, crush phenomenon that can appear on obtained tissue samples during bronchoscopy and inadequate fixation of them can put pathologist on misdiagnosis.

1.4. Routine treatment of biopsy samples when there is a suspicion of NSCLC

Biopsy samples are routinely fixed in a 10% buffered formalin and then dehydrated in xylol and rising alcohol concentrations, embedded into paraffin block, cut at thickness of 2 μm, using hematoxylin-eosin stained, covered with medium (Canada balsam) and analyzed under the microscope. There should be only one, two at most cross sections, on one object plate in order to determine whether there is cancer in the first two cross sections by routine hematoxylin-eosin (H&E) staining and also to assume a histological type of cancer, based on the morphological char-acteristics of malignant cells. The following sections are cut separately on two respective plates for immunohistochemical staining in order to determine the precise histological type of NSCLC. The pathologist concludes that there are no malignant cells on both cross sections and to report this in his definitive pathohistological finding. If NSCLC is found, two immunohistochemical stainings are applied, TTF-1 and p40, which determine the histological subtype of two most histological subtypes of NSCLC, adenocarcinoma and squamous cell carcinoma. In the end, it is desirable for the pathologist to indicate in his report whether there is enough and how much tissue material is left for the next molecular testings. The number of plates is 4: for EGFR molecular testing and ALK, ROS1 and PDL-1 immunohistochemical evaluation (**Figure 2**). In the era of personalized therapy, it is desirable to cut eight tissue sections at the same time from paraffin block: two for morphological analysis based on H&E stained preparations, two for basic immunohistochemical staining (TTF-1 and p40) and the last four for molecular and immunohistochemical predictive staining only in the case that NSCLC is found on H&E stained cross sections [12, 13].

2. Immunohistochemistry

2.1. Immunohistochemical staining method

The labeled streptavidin-biotin staining method uses a highly "refined" avidin-biotin complex (ABC) three-stage technique in which a biotinylated secondary antibody reacts with several streptavidin molecules conjugated by peroxidase or alkaline phosphatase [12].

2.2. Immunohistochemical staining procedure

Tissue samples for immunohistochemical staining are deparaffined according to the prescribed procedure of the manufacturer and then incubated with a specific serum at room temperature in a damp chamber for a prescribed duration. It is used the labeled streptavidin-biotin (LSAB) technique. The antigen-antibody complex is visualized by 3-amino-9-ethylcarbazole or diaminobenzidine hydrochloride solution. Mayer's hematoxylin is used as a counterstain. A "positive control" is used to evaluate the effectiveness of a method or reaction.

As already stated, "internal positive control" is used, since there are normal tissue structures on the preparation itself, in this case, lung, which express the administered antibodies. In the part of the chapter in which we analyze individual monoclonal antibodies, we will also indicate which lung structures are expressing them regularly [12, 14, 15].

3. Monoclonal antibodies in the diagnosis of non-small cell lung cancer

There is a question, which two monoclonal antibodies should be rationally applied in order to establish the exact diagnosis of the histological subtype of NSCLC. Currently, these are thyreoid-transcription-factor-1 (TTF-1) and p40. These two antibodies have a role in distinguishing two most common histological subtypes, adenocarcinoma and squamous cell carcinoma. TTF-1 (clone 8G7G3/1, DAKO Cytomation, Denmark) is a diagnostic marker for adenocarcinoma of the lung and p40 (BC28, Ventana, USA) for squamous cell carcinoma [16, 17].

However, in the past, other less specific antibodies were used in the differentiation of histological subtypes of NSCLC. They can also be used now as an additional confirmation about histological subtype of NSCLC and differentiation of primary from secondary lung cancer. The following antibodies are also useful for differentiation: NapsinA (clone IP64, Novocastra™ HD, Leica Biosystems, UK), p63 (clone 7JUL, Novocastra™ HD, Leica Biosystems, UK), Cytokeratin5/6 (cloneD5/16B4 DAKO Cytomation, Denmark) or Cytokeratin5 (clone EP1601Y, Cell Marque RUO, USA) and Cytokeratin7 (clone OV-TL 12/30 DAKO Cytomation, Denmark) [18, 19]. If the morphological picture of NSCLC has organoid appearance, there is a suspicion that this is large cell lung carcinoma with neuroendocrine differentiation (LCC-NEC). To confirm this suspicion, it is necessary to use at least one of two neuroendocrine markers, CD56 (clone NCAM-1 Ab-2, Thermo Scientific LabVision, USA) or Synaptophysin (clone 27G1 Novocastra ™ HD, Leica Biosystems, UK) [18, 20]. If TTF-1, p40 and optionally CD56 were not expressed, for the purpose of storing malignant tissue for molecular testing, it should

be concluded that this is non-small-cell lung carcinoma, or "not otherwise specified" (NOS) carcinoma [7, 16]. It should be noted that no antibody is not highly specific for one organ or one histological type of cancer, that is, each antibody is specific for more than one histological types of cancer or more organs [21].

For precise pathohistological diagnosis of histological subtype of NSCLC, it is necessary to use two antibodies, TTF-1 and p40. If it is estimated that there is enough tumor tissue in a paraffin mold, it is possible to use additional antibodies to confirm the diagnosis and to preserve it for molecular testing [22, 23].

The advantages and disadvantages of each of these antibodies are discussed in the next section.

3.1. Thyroid transcription factor-1 (TTF-1)

Thyroid Transcription factor-1 (TTF-1) is a nucleic specific protein transcriptional factor expressed by thyroid gland and thyroid tumors as well as adenocarcinoma of the lung (**Figures 3** and **4**). This marker is expressed in the majority of lung adenocarcinoma (75%), but also in about 10% of squamous cell carcinoma. TTF-1 is significant in the differential diagnosis between primary and metastatic adenocarcinoma. At a time, when less antibodies were known, if TTF-1 with Cytokeratin7 was positive and Cytokeratin20 negative, diagnosis of adenocarcinoma of the lung was established [5, 24–28]. Diagnostic algorithms in the diagnosis of adenocarcinoma and differentiation of adenocarcinoma from squamous cell carcinoma are shown in papers of IASLC/ATS/ERS and Terry et al. [29, 30]. In various studies, TTF-1 was specific from 88 to 97% [6, 30, 31]. This marker is also expressed in lung cancer with neuroendocrine differentiation, in typical and atypical carcinoids, as well as in more than 90% of small cell lung carcinomas with neuroendocrine differentiation and in 50% of large cell lung carcinomas with neuroendocrine differentiation [30]. Literature reveals that TTF-1 can be expressed in breast and ovarian carcinoma [31, 32].

In one of our studies, we found that TTF-1 was expressed in 85.2% and in the other study, in 86% of lung adenocarcinomas [12, 33]. We also found that TTF-1 was also expressed in benign lung tumors [34, 35].
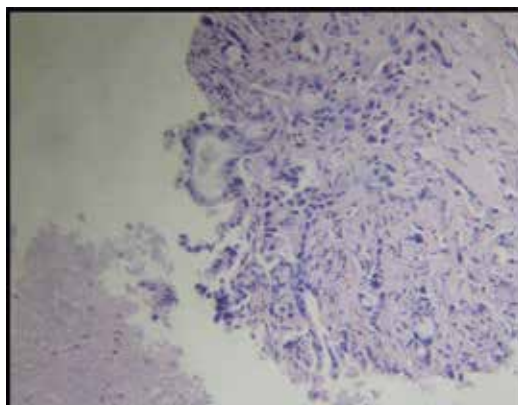
Figure 3. Adenocarcinoma of the lung with acinar growth, fnab, H&E 100×.
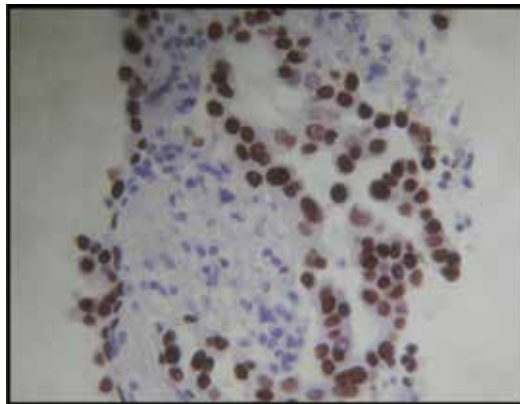
Figure 4. Adenocarcinoma of the lung, nucleic expression, TTF-1 200×.

3.2. p40

Role of p40 is to distinguish adenocarcinomas from squamous cell carcinomas in small samples (**Figure 5**) as well as on cytological smears. Squamous cell carcinoma is confirming with p40 antibody which is also known as DNp63. p40 is expressed in the nucleus of malignant cells of squamous cell carcinoma (**Figure 6**). This antibody is more specific for squamous cell lung carcinoma from p63. p40 is expressed in a smaller number of lung adenocarcinoma cells than p63. That is why it is recommended to use p40 instead of p63 for diagnostics of squamous cell carcinoma [36, 37]. If in malignant cells of carcinoma are not expressed TTF-1 and p40, diagnosis of non-small cell lung carcinoma -not otherwise specified (NSCLC-NOS) on a small biopsy sample will be established [16].

However, p40 is not a highly specific marker for only squamous cell lung carcinoma. It is also highly specific marker for urothelial carcinoma. This means that metastatic urothelial carcinoma in the lung is difficult to distinguish from primary squamous cell lung carcinoma both for morphological similarity these two carcinomas and similar immunophenotype [38]
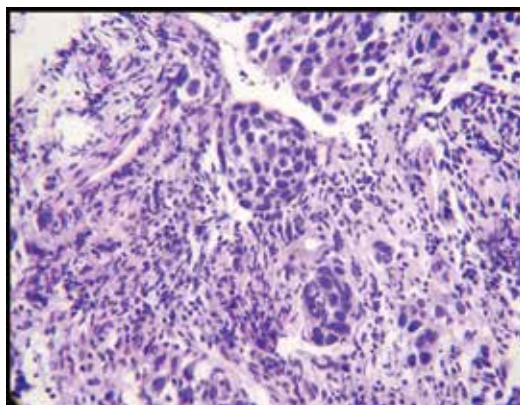
Figure 5. Moderately differentiated squamous cell carcinoma of the lung on bronchoscopic biopsy, H&E 20×.
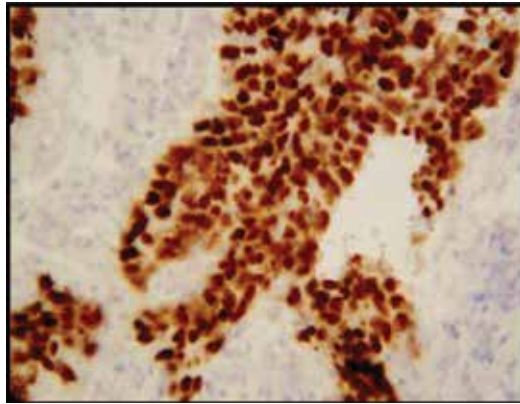
Figure 6. p40 expression in the squamous cell lung nuclei, 400×.

(**Figures 7** and **8**). Therefore, for definitive differentiation lung squamous cell carcinomafrom urotothelial carcinoma is also required clinical data on current local status in previously operated patients with urothelial carcinomas (degree of cancer invasion at the time of surgery, presence of angioinvasion, state of resection margins, the presence of metastases in local and remote lymph nodes).

p40 is also expressed in squamous cell carcinoma of other localizations (head and neck, larings, trachea, cervix, skin). That is why it is not possible to differentiate primary squamous cell lung carcinoma from metastatic lung carcinoma of the same histological type by using p40. Differentiation of primary from secondary squamous cell carcinoma in the lung is possible only in clinicopathological correlation [39].

3.3. NapsinA

NapsinA is expressed in the cytoplasm of preserved lung parenchyma in the form of functional aspartic proteinase, homologous to the polypeptide Tao2 and included in maturation of the
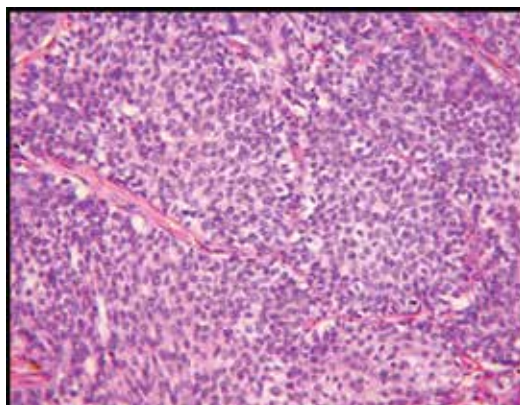
Figure 7. Urothelial carcinoma metastases in the lung, 400×.
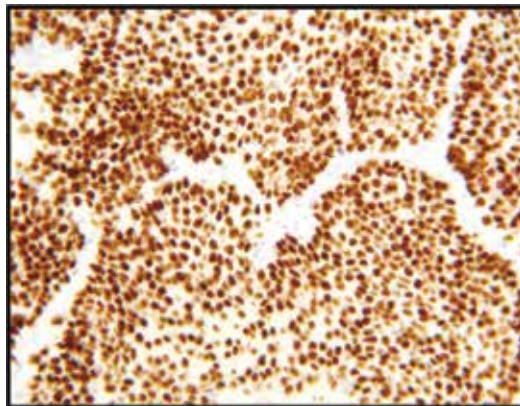
Figure 8. p40 expression in urothelial carcinoma metastases in the lung, 400×.

biologically active surfactant protein B. It also consists of 38-kDa protein, protein chain which is expressed in type II pneumocytes, alveolar macrophages and renal tubules, secretion channels of exocrine glands and pancreas. NapsinA is staining as coarse-grained intracytoplasmic marker [40].

There are papers that favor the use of NapsinA in regard to TTF-1 in differentiation of lung adenocarcinoma from other histological subtypes of NSCLC. Papers were done on a large number of lung adenocarcinomas wherein NapsinA showed higher specificity compared to the TTF-1. It is even recommended double immunohistochemical staining, so-called cocktail, which would beside NapsinA for cytoplasmic staining, contain and p40 for nuclear staining for squamous cell lung carcinoma [17, 36, 40, 41]. A simple diagnostic algorithm from 2010 recommends that NapsinA should be applied in order to diagnose adenocarcinoma, if TTF-1 is not exposed in NSCLC and if squamous cell carcinoma is not proved [30]. Our study on 50 adenocarcinomas showed a greater specificity of TTF-1 as compared to NapsinA [34]. If NapsinA is not expressed and the presence of mucin has not been proven, it should be concluded that it is about NSCLC-NOS. The definitive conclusion is that NapsinA is supplemental to TTF-1 which serves as the main marker for proving of lung adenocarcinoma (**Figure 9**).

3.4. p63

This antibody is known in the two isoforms, DNp63 and TAp63. It is expressed in the nucleus of the malignant cells. According to previous diagnostic algorithms, before occurrence of p40, p63 was the main marker in the differentiation of NSCLC on small biopsy samples beside TTF-1 [29]. Beside TTF-1, NapsinA and Cytokeratin5/6 are considered to be useful markers for the diagnosis of NSCLC on small biopsy samples [28]. The degree of p63 expression increases with the decrease in the degree of keratinization, that is, differentiation of squamous cell carcinoma [25] (**Figure 10** 200×; **Figure 11** 400×).

However, p63 as well as p40 is not a highly specific marker only for squamous cell carcinoma. It is also a marker for urothelial carcinoma. That is why it is hard to differentiate metastatic carcinoma of urothelial type from primary squamous cell lung carcinoma [39] (**Figure 12** 200×; **Figure 13** 400×).
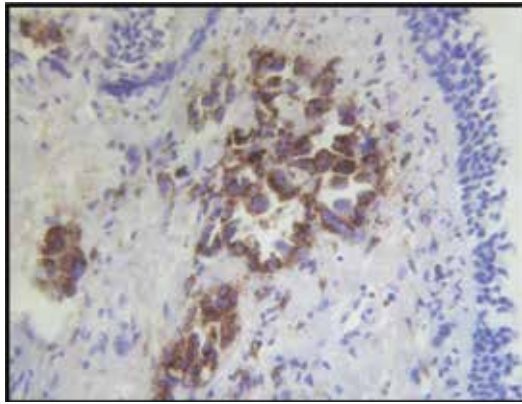
Figure 9. Bronchoscopic biopsy, infiltrate of the acinar adenocarcinoma of the lung in the mucosallayer of the bronchi, proven by using NapsinA 200×.
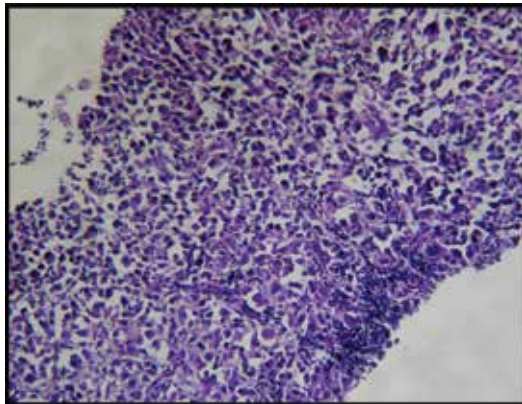
Figure 10. NSCLC deposit in the lymphatic tissue, where squamous cell carcinoma was immunohistochemically proven, H&E 200×.
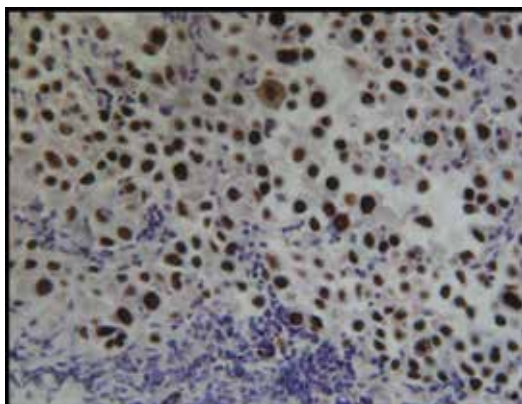
Figure 11. Strong nuclear expression of p63 in the cells of poorly differentiated squamous cell lung carcinoma, 400×.
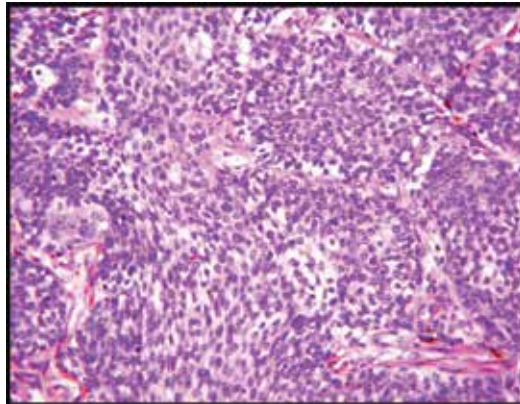
Figure 12. Urothelial carcinoma with lung metastases, H&E 400×.
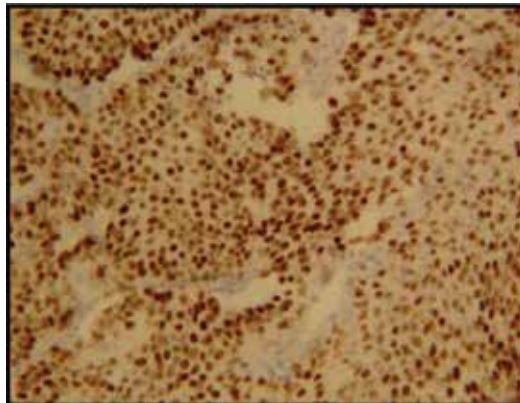
Figure 13. p63 expression in urothelial carcinoma with lung metastases, 400×.

3.5. Cytokeratin5/6 or Cytokeratin5

Cytokeratin 5/6 or Cytokeratin5 is a cytoplasmic marker. Its expression is present at squamous cell carcinoma (**Figure 14**). Because of that, this marker can be used as a confirmation for this histological type, together with p40 and p63, especially if there are no technical conditions for using one of these antibodies. Except in the regular epithelium of bronchial airways, Cytokeratin 5/6 or Cytokeratin5 is also expressed in regular and reactive mesothelioma cells, but also in the epithelium cells of the malignant pleural mesothelioma. Ovarian carcinomas, especially those of serous type, are cytoplasmically expressed antibody [21, 28, 42].

3.6. Neuroendocrine markers

According to WHO recommendations about the classification of lung carcinoma from 2004, in order to establish the diagnosis of large cell lung carcinoma with neuroendocrine differentia-tion, it is necessary for the cells to be large and round in an organoidal arrangement, with a
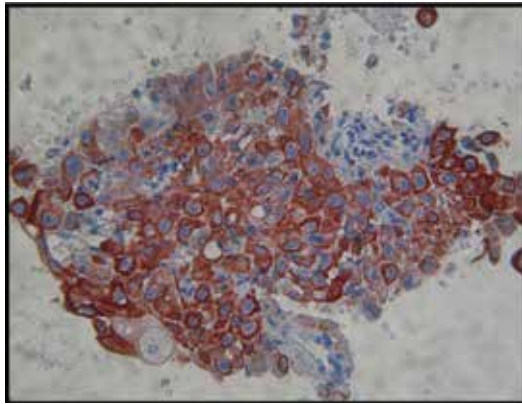
Figure 14. Expression of Cytokeratina5/6 in squamous cell carcinoma, fnab, 200×.
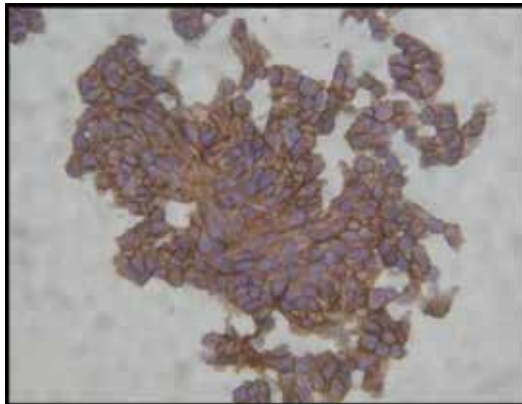
Figure 15. Membrane expression of CD56 into LCLC-NEC cells, 200×.
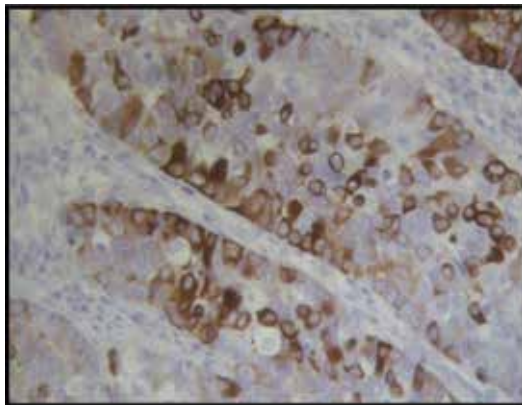
Figure 16. Cytoplasmic expression of Synaptophysin into NSCLC with partly adenoid growth pattern 200×.
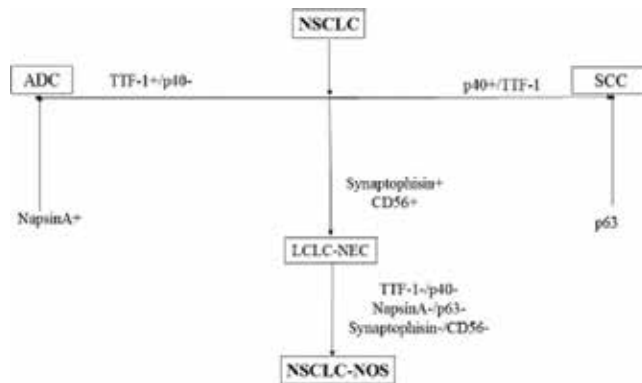
Figure 17. The proposed algorithm for the differential diagnosis of non-small cell lung carcinoma by using immunohistochemical stainings.

luminous and large nucleus and noticeable nucleolus and to express at least one neuroendocrine marker: CD56 (**Figure 15**), Synaptophysin (**Figure 16**) or ChromograninA [5, 6]. It means that if on a small biopsy sample, non-small cell lung cancer is showing organoid, trabecular or acinar growth pattern, and two neuroendocrine markers, preferably CD56 and Synaptohysin [43], should be used to confirm this type of cancer (LCLC-NEC) (**Figure 17**).

4. Discussion

Finally, someone may ask why it is necessary to know of which histological type of non-small cell lung cancer is about and to save enough malignant tissue in paraffin block for molecular testings at the same time.

The answer is that it is necessary to know how to apply certain tests for the application of personalized oncology therapy. If the evaluation of the tests showed that a positive response on therapy is expected, the same will be applied.

Globally, adenocarcinoma of the lungs is the most common histologic type of lung cancer. EGFR molecular testing is used to assess the response to tyrosine kinase inhibitors (TKI) therapy in patients with adenocarcinoma. Positive response to the TKI therapy is more commonly expected in young nonsmoking woman. It is also recommended that this testing is done in young nonsmoking woman with squamous cell lung cancer because it showed positive results. The result of this molecular testing depends on the sensitivity of the method and number of cells remaining after morphological and immunohistochemical diagnostics, more than 5% of malignant cells in relation to the total number of cells on the cross section. EGFR mutations are diagnosed in 10–15% of Caucasians patients with NSCLC [22, 44].

ALK testing (clone D5F3, Ventana, USA) is done in patients with adenocarcinoma of the lungs in which EGFR mutations have not been detected. There is no consensus on whether this test should be performed only in lung adenocarcinoma or it can be performed in all

pathohistological subtypes of NSCLC. Namely, there are several diagnostic algorithms which include all histological types of NSCLC or only adenocarcinoma of the lungs. There are data indicating that this genetic rearrangement was detected only in about 4% of patients with adenocarcinoma of the lungs or in about 2% of patients with NSCLC. In order for this test to be valid, it is necessary that on the tissue cross section at least 50 malignant cells have to be present after primary diagnostics and EGFR testing. Then immunochemical testing would be valid and where there is this type of consensus, to confirm the presence of rearrangements, FISH test should also be done [13, 45, 46].

It is necessary to know the fact that about 70% of LCC-NEC is ALK positive, but that its expression is not in correlation with personalized ALK inhibitors. If NSCLC have an organoid morphological picture, it is necessary to apply CD56 and Synaptophysin on small biopsy samples in order to exclude LCC-NEC [13, 45, 46].

ROS1 testing (clone D4D6, Ventana, USA) is also done in adenocarcinoma of the lungs where EGFR and ALK tests did not show positive results. This fusion is present only in 1–2%, predominantly younger patients, nonsmokers, with adenocarcinoma of the lungs. The presence of fusion is immunohistochemically determined, and it is necessarily confirmed by the FISH method, so it is necessary to preserve tissue for these methods after diagnosis of adenocarcinoma [13, 46].

If any of these tests fail to give results, for the application of immunotherapy, it is applied PDL-1 testing (clone 22C3, DAKO Cytomation, Denmark), mainly in squamous cell lung cancer, but also in adenocarcinomas in which previous test did not show positive results. For this immunohistochemical testing, it is recommended that tissue cross section contains at least 100 malignant cells [47, 48].

The future of personalized lung cancer treatment is next-generation sequencing (NGS), polymerase chain reaction (PCR) method, in which, from the remaining of paraffin block in which NSCLS was diagnosed, at the same time is detecting all druggable mutations [23].

Liquid biopsy is a method that detects DNA tumor cells in whole or in its parts. This method is useful in early detection of lung cancer, but it is false negative if circulating malignant cells do not possess a mutation [49].

5. Conclusions

Precise diagnosis of NSCLC histological type is based only on morphological characteristics of the tumor, cell appearance and their growth pattern on H&E stained preparation is rarely possible. Based on morphological findings, keratinizing squamous cell carcinoma can be diagnosed with great certainty, without immunohistochemistry. The main disadvantage of all used antibodies is not highly specificity only for one histological type of cancer or originates from more than one organ. In order to preserve tumor tissue for molecular testing, one must use only one, the most specific antibody for one histological type of carcinoma. For adenocarcinoma of the lung, highly specific is TTF-1, and for squamous cell carcinoma, it is p40. Other

additional antibodies can be applied only if there is a greater amount of bioptic tumor material (*fnab* biopsy or *VATS* biopsy). NapsinA is an additional antibody, which can be applied in proving adenocarcinoma of the lung and p63 for squamous cell carcinoma. When LCLC-NEC is suspected, it is recommended to use only one neuroendocrine marker, and the most reliable is CD56. For saving, in order to apply all the appropriate antibodies and to preserve tissue for molecular testing, it is necessary to cut only one cross section on one plate.

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Paraneoplastic Syndromes in Lung Cancer

Dilaver Tas

Additional information is available at the end of the chapter

Abstract

In recent years, the incidence of lung cancer (LC) has been increasing throughout the world and is the most common type of cancer in all regions of the world, occurring more frequently in men than in women. Paraneoplastic syndromes (PNS) refer to clinical conditions that develop in relation to tumors, without physical effects of the primary or metastatic tumors. The development of PNS is not associated with the size of the primary tumor or the extent of metastases. It is usually seen in small-cell lung cancer (SCLC) as well as other types of lung cancer. PNS developed in almost 1 in 10 patients with lung cancer and it may be an indicator for the diagnosis of lung cancer and it can be seen during later stages of cancer or at the time of cancer recurrence. Accordingly, the identification of these syndromes can be helpful in the early diagnosis of occult cancers, allowing timely treatment. PNS decreases the quality of life of the patients with cancer and thus requires specific treatment. Moreover, these conditions can be used as a marker of cancer activity and can predict prognosis. In this section, a detailed description of PNS is provided.

Keywords: lung cancer, small-cell lung cancer, non-small-cell lung cancer, paraneoplastic syndromes

1. Introduction

1.1. Definition

The term "paraneoplastic syndrome (PNS)" refers to tumor-related symptoms and findings that are independent of the direct, local extent or physical effects of metastases. PNS develops in response to the effects of hormones and cytokines released from cancer cells, or due to the immunologic response of cancer cells [1, 2]. In this regard, there is no single mechanism

underlying the development of PNS, and potential mechanisms have not yet been clearly understood. On the other hand, several tumor-secreted proteins that may be associated with the development of PNS have been defined in the recent years. Generally, the ectopic production of peptide hormones with hormonal activity and immunological mechanisms can be seen in patients with PNS.

The diagnosis and treatment of PNS are complementary parts of lung cancer (LC) management. PNS may involve several organs and systems, and so may therefore result in neurological, dermatological, hematological, nephrological, rheumatologic, metabolic, immunologic and constitutional signs and symptoms.

1.2. History

In 1865, Armand Trousseau, a French internal diseases specialist, stated that the identification of unexpected or migratory thrombophlebitis could indicate an occult visceral malignancy [3], and today, the development of superficial migratory thrombophlebitis related to visceral cancer is known as Trousseau syndrome. An Austrian dermatologist, Ferdinand von Hebra, underlined the significance of internal disease in the etiology of several skin manifestations such as urticaria, generalized pruritus, xanthoma, and pemphigus. In 1868, he further stated that skin pigmentation could be an indicator of cervical cancer [4, 5]. In 1890, a French physician named Auche defined peripheral nervous system involvement in patients with stomach, pancreas, and uterus cancer [6], while acanthosis nigricans associated with malignancy was reported separately by Pollitzer and Janovsky in 1890 [7]. Later, Brown identified the Cushing syndrome in a patient with small-cell lung cancer (SCLC) in 1928 [8], and in 1933, neuropathy development was reported in a patient with oat cell carcinoma (small-cell lung cancer) [9]. Guichard and Vignon used the term "paraneoplastic" for the first time in 1949 when they identified central and peripheral neuropathies in a patient with cervical cancer [10]. In 1957, Schwartz et al. reported hyponatremia in a patient with LC [11]. In 1967, Bartter and Schwartz defined the syndrome of inappropriate antidiuretic hormone secretion (SIADH) [12]. The relationship between the neurological PNS and LC was first suggested by Oppenheim at the end of the nineteenth century [9].

In the following years, etiologic and pathogenetic studies were carried out to evaluate the relationship between PNS and cancer including LC. Additionally, new PNS definitions and new developments have been made which are still ongoing.

1.3. Epidemiology

LC is currently the most common type of cancer throughout the world [13]. Due to the high incidence rate of LC and the relatively high frequency of PNS in SCLC cases, LC-related PNS is more common than other types of cancer [14–17]. However, it is difficult to estimate PNS frequency due to challenges in the identification of PNS and uncertainty in the differentiation of its symptoms because of the underlying disease. PNS can be seen in all age groups of patients with cancer. However, due to the nature of cancer, it is more common in middle-aged and older individuals. PNS develops in 1–7.4% of all patients with cancer, although it is estimated to develop in 20% of all patients with cancer [14]. The inclusion of generalized malignancy symptoms, such as cachexia and fever, increases PNS frequency up to 70% [18].

PNS can occur both in patients with SCLC and with non-small-cell lung cancer (NSCLC). SCLC has a neuroendocrine origin and PNS is more common in such cancer types. PNS is seen by 7–15% of all patients with LC. Systemic symptoms and findings of PNS develop in 50% of patients with SCLC and almost 10% of patients with NSCLC [19]. PNS develops secondary to LC and increases the severity of the disease. It is therefore crucial to recognize PNS in these patients.

Incidences of PNS will increase in the coming years thanks to the improvements in diagnosis and treatment of LC as well as in the diagnosis of PNS.

1.4. Pathophysiology

PNS may develop under the effects of substances released by the tumor or as a result of the cross-reactions between tissues and the antibodies produced against the tumor.

Studies investigating the pathogenesis of PNS offer some evidences that PNS develops based on different pathogenetic mechanisms, such as:

1. The production of special substances by tumor cells, leading specifically to the development of PNS. These substances may be hormones, growth factors, vasoactive peptides, cytokines, enzymes or other signaling molecules.

2. Abnormal immune response of the host organ to the neo-antigens produced by the tumor or to other tumor products [7, 18, 20].

Endocrinologic PNS generally develops due to the increased production of hormones or hormone precursors by malignant cells. The best example for this is paraneoplastic Cushing syndrome seen in patients with SCLC [21].

Paraneoplastic hypercalcemia is an example of PNS associated with cytokine production. Some cytokines (IL-1, 3, and 6, prostaglandins, TGF-α, TNF-α [lymphotoxin], and TNF-β [cachectin], etc.) that are synthesized by the malignant cells may result in hypercalcemia by activating osteoclasts [22].

Cancer cells are recognized by the immune cells and lead to the production of antibodies. As cancer cells are identical to normal cells in nature, the antigens on the cancer cells are similar to those of natural cells. Therefore, the formed antibodies may have a cross-reaction with normal tissues. This pathophysiological condition is most commonly seen in neurological PNS [23, 24].

The main mechanism underlying above-described pathological response in patients with LC and other types of cancer still presents an unanswered question. The most appropriate answer to that would be inappropriate gene expression (IGE). IGE may be described as the formation of an inappropriate gene programmed to produce tumoral proteins in cancer cells. This may lead to the development of new disorders that will negatively affect patient well-being. Thus, the quality of life of the patient is impaired and the severity of the disease increases [18].

Genetic studies that will be performed in the future to identify IGEs in cancer patients will allow the early detection of PNS, even before the clinical diagnosis, and more importantly, will be helpful to identify malignant formations.

2. Paraneoplastic syndromes

The diagnosis of PNS is relatively challenging, as lesions may develop in regions distant to the cancer and may not resemble a cancer-related disease, and the disorder has benign forms in general as well as malignant forms. PNS should be suspected in the presence of below characteristics: absence of a defined etiology for the associated syndrome; correlation between the time of diagnosis of the syndrome and that of cancer; clinical and histological remission after complete surgical or chemotherapy treatment and a worsening of symptoms due to tumor residue [25].

Although PNS may be associated with a lot of malignancies, they are associated most commonly with lung cancer, specifically SCLC. Humoral hypercalcemia and SIADH, which is seen in orderly squamous cell and SCLC, are the most common PNSs. Multiple paraneoplastic syndromes can be seen in patients with SCLC. In the literature, there have been patients with two or more paraneoplastic syndromes associated with SCLC, even though rarity. Paraneoplastic syndromes usually have a course parallel to the underlying malignancy. Treating the underlying tumor is the first choice and symptomatic therapy can be useful [2, 26–29].

2.1. Classification

Paraneoplastic symptoms in LC can be seen almost in all systems, which can be listed as follows:

- Endocrine paraneoplastic syndromes

- Neurologic paraneoplastic syndromes

- Dermatologic paraneoplastic syndromes

- Rheumatologic paraneoplastic syndromes

- Nephrological paraneoplastic syndromes

- Hematologic paraneoplastic syndromes

- Others

2.2. Endocrine paraneoplastic syndromes

Endocrinological PNS generally develops due to excessive synthesis of hormones or hormone precursors with low bioactivity, or the conversion of the precursors to more effective product(s) in the tumor tissue.

2.2.1. Ectopic Cushing syndrome

Secretion of ectopic adrenocorticotropic hormone (ACTH) from the tumor may result in Cushing syndrome, and high ACTH levels can be noted in almost 50% of patients with LC. Cushing syndrome may develop in 1–5% of patients with SCLC [30, 31], and of all the pulmonary

neuroendocrine tumors, about 1–2% are accompanied by Cushing syndrome due to ectopic ACTH secretion, or the production of its precursor, proopiomelanocortin (POMC) [32].

Patients are typically admitted to clinics due to myopathy, centripetal obesity, facial plethora, hypertension, osteoporosis, hyperglycemia, hirsutism, and acne. The clinical symptoms have a rapid onset and are generally accompanied by hypokalemia and hyperglycemia.

Due to its rapid onset, patients generally are found to have electrolyte imbalances rather than having a cushingoid appearance, although typical cushingoid characteristics such as moon face and buffalo hump generally do not manifest, as hypercortisolism is an acute phenomenon and patients do not live long enough for the manifestation of morphological changes [33, 34].

Increased levels of free cortisol in the urine, elevated serum levels of ACTH or ACTH precursors, hypokalemia and hyperglycemia are helpful for the diagnosis of ectopic Cushing syndrome (ECS).

The prognosis of SCLC patients with Cushing syndrome is worse than in SCLC patients without Cushing syndrome [35].

Treatment essentially involves treating the primary tumor. In the presence of non-resectable tumors, medications that inhibit steroid biosynthesis (metyrapone, ketoconazole) can be used for the treatment of ECS. Aminoglutethimide can be used to prevent androgenic side effects and octreotide may be helpful in reducing ACTH release. A bilateral adrenalectomy may also be considered [31].

2.2.2. Inappropriate antidiuretic hormone syndrome

Inappropriate antidiuretic hormone syndrome (IADHS) develops as a result of free water retention and increased extracellular fluid volume due to the irregular release of the antidiuretic hormone (ADH). This initiates a process characterized by a progressive dilution of plasma sodium and sodium loss through the kidneys.

It is generally seen in patients with SCLC, accounts for around 75% of all cancer-related IADHS. It is seen in 7–16% of patients with SCLC but may also occur in patients with NSCLC [36, 37].

Antidiuretic hormone (ADH) levels are generally elevated in LC patients with IADHS and in addition, patients may also have increased levels of atrial natriuretic peptide [38].

IADHS symptoms rarely manifest when plasma sodium levels are higher than 125 mEq/L. Plasma sodium levels below 125 mEq/L may result in symptoms such as weakness, tiredness, nausea, headache, lethargy, and confusion, while levels below 120 mEq/L may lead to seizure and coma [39].

Based on the definition of Bartter and Schwartz, a diagnosis of IADHS can be made in the presence of following findings:

- Serum Na < 134 mEq/l

- Plasma osmolality <275 mOsm/kg

- Urine osmolality >500 mOsm/kg

- High urinary sodium concentration (>20mEq/l)

- Absence of clinical signs of volume depletion

- Presence of normal adrenal functions

- Presence of normal thyroid functions [1, 12]

- Presence of IADHS is a marker of poor prognosis [38]

If plasma sodium levels are higher than 130 mEq/L, fluid intake may be limited (500 mL/day) and/or patients may be given demeclocycline, which is a medication that blocks the response of renal tubules to ADH. A slow infusion of hypertonic saline solution and an IV furosemide infusion may be preferred in severe cases [31].

2.2.3. Hypercalcemia

Hypercalcemia develops in 6% of LC patients, and is most commonly seen in the presence of squamous cell carcinoma [40, 41]. In LC, hypercalcemia is caused by the production of parathyroid hormone-related protein (PTHrP) of the tumor and secretion of parathyroid hormone. Another mechanism leading to the development of hypercalcemia involves PTHrP increase as a result of granulocyte colony-stimulating factor signal. While increased $1.25(OH)_2$ Vitamin D synthesis may result in hypercalcemia in some malignancies, this mechanism is not associated with hypercalcemia in LC [41].

Early symptoms of hypercalcemia include loss of appetite, nausea, vomiting, constipation, numbness, polyurea, polydypsia, and dehydration, while late symptoms include renal failure, nephrocalcinosis, confusion, and coma.

Elevated serum levels of ionized calcium, normal or decreased levels of parathyroid hormone and high PTHrP levels are diagnostic factors [9].

Treatment is essentially based on the treatment of the tumor-causing hypercalcemia. Symptomatic patients with high serum calcium levels should be treated with hydration and bisphosphonates, and any calcium supplements, thiazide diuretics, and lithium, all of which may alleviate hypercalcemia, should be discontinued [42].

2.2.4. Other endocrine paraneoplastic syndromes

In LC patients, hypoglycemia may occur due to ectopic insulin secretion and insulin-like growth factor (IGF) secretion.

Acromegaly may also occur as a result of ectopic growth hormone-releasing hormone (GHRH) or IGF secretion.

Moreover, carcinoid syndrome may develop in relation to the secretion of serotonin and similar vasoactive amines [41, 43].

2.3. Neurologic paraneoplastic syndromes

Neurologic PNS may develop by the involvement of central nervous system, peripheral nervous system or the neuromuscular junction and muscles. The majority of patients with neurologic PNS have SCLC (at a rate of 5%). In its pathogenesis, immune-system mediated reactions are seen in general. Immune cross-reactivity is seen between tumor cells and components of the nervous system [9, 29, 44].

International criteria have been determined to facilitate the diagnosis of paraneoplastic neurologic syndromes, defining "definite" and "possible" diagnoses. The definite diagnostic criteria are as follows:

- neurological symptoms that will develop cancer within 5 years and commonly accompanying with cancer (limbic encephalitis, cerebellar degenerations, etc.);

- nonclassical neurologic syndrome that improves after cancer treatment without concomitant immunotherapy;

- nonclassical neurologic syndrome with positive antibodies and a diagnosis of cancer within 5 years; and

- neurologic syndromes accompanied by "well-characterized" antibodies in the absence of a cancer diagnosis (anti-Hu, anti-CV2, anti-Ri, anti-Yo, anti-Tr, and anti-Ma2).

There are three categories of possible paraneoplastic neurologic syndrome diagnoses:

- presence of classical neurologic syndrome in the absence of cancer or antibodies, but a high risk of underlying tumor;

- presence of neurologic syndrome with non-classical antibodies in the absence of cancer; and

- presence of classical neurologic syndrome without antibodies or a malignancy [9, 45].

2.3.1. Encephalomyelitis

Paraneoplastic encephalomyelitis should be considered in the presence of neuronal loss and inflammation in multiple regions of the central nervous system, primarily in the hippocampus (limbic encephalitis), Purkinje cells of the cerebellum (cerebellar degeneration), brainstem (brainstem encephalitis) and medulla spinalis (myelitis). Dorsal root-ganglion (sensorial neuronopathy), as well as sympathetic and parasympathetic nerve and ganglions (orthostatic hypotension, gastrointestinal paresis, arrhythmia, erectile dysfunction) are known to be involved in the majority of the cases [46, 47].

It is thought to be developed with SCLC and due to the immune response that develops against the neural proteins expressed by the tumor. The most commonly identified antibody in paraneoplastic encephalomyelitis is the Hu (ANNA-1) antibody, although cases associated with CV2, amphiphysin, and Ri antibodies have also been reported.

While it may start with relatively milder and focal signs such as epilepsia partialis continua, nonconvulsive epileptic status or frontal-type ataxia, the findings rapidly progress within weeks or months and may result in death in general. Neurological outcomes may not be satisfying despite the treatment, as irreversible neuronal damage occurs in most of cases when the diagnosis is made. The worst neurological outcomes are seen in paraneoplastic encephalomyelitis accompanied by Anti-Hu antibodies [48].

In addition to cancer treatment, immunomodulation plays a key role in the treatment of encephalomyelitis, and corticosteroids, steroid-sparing agents (azathioprine, cyclophosphamide, etc.), rituximab, IVIG, and plasmapheresis can be used for treatment [44].

2.3.2. Limbic encephalitis

Paraneoplastic limbic encephalitis manifests with major findings such as short-term memory loss, behavior/mood changes and epileptic seizures, and confusion, irritability, depression, sleep problems, hallucinations and psychosis in addition to major findings [49]. Moreover, hyperthermia and endocrine disorders may also develop due to hypothalamic dysfunction. Antibodies produced against the Hu, Ma2, CV2 and amphiphysin antigens expressed by the tumor have been identified in patients with paraneoplastic limbic encephalitis. For the diagnosis, demonstration of epileptic activity in the electroencephalographic examination, demonstration of temporal lobe involvement in magnetic resonance imaging (MR2), and an investigation of the cerebrospinal fluid (CSF) and auto-antibody test are required. The neurologic response to treatment varies between patients with paraneoplastic limbic encephalitis, while 30–50% of patients may recover after the tumor treatment.

Just like in encephalomyelitis, immunomodulation plays a key role in treatment along with cancer-specific therapies, and corticosteroids, steroid-sparing agents (azathioprine, cyclophosphamide, etc.), rituximab, IVIG, and plasmapheresis can be used for treatment [44].

2.3.3. Cerebellar degeneration

Paraneoplastic cerebellar degeneration is one of the most common PNS. Depending on the widespread cerebellar Purkinje cell death, it may have an acute or subacute onset and courses with a rapidly progressing pancerebellar syndrome. The antibodies associated with cerebellar degeneration include anti-Yo, Tr, Hu, Ma2, and Ri [50]. However, the most well-defined and frequently seen paraneoplastic cerebellar degeneration is associated with the Yo antibodies. Before developing findings of neurologic deficit, patients may experience flu-like prodromal signs and may then develop an ataxic gait, dysarthria, dysphagia, diplopia, blurred vision, and transient opsoclonus. Patients initially have normal MR and CSF findings, however, inflammatory findings in CSF develop rapidly and an MR may demonstrate advanced cerebellar atrophy. Fast and effective treatment may prevent the progression of symptoms, but cerebellar degeneration is one of the most treatment-resistant PNS.

Like in limbic encephalitis, treatment may include the use of corticosteroids, steroid-sparing agents (azathioprine, cyclophosphamide, etc.), rituximab, IVIG, and plasmapheresis [44].

2.3.4. Lambert-Eaton myasthenic syndrome

Almost 60% of the Lambert-Eaton myasthenic syndrome (LEMS) cases have a paraneoplastic origin. SCLC is observed in the vast majority of cancer cases with LEMS, while other types of cancer are seen in a small number of cases [51]. LEMS develops in 3% of all SCLC cases, and LEMS develops as a result of the autoimmune response to the P/Q-type anti-voltage-gated calcium channels (VGCC) that exist on the pre-synaptic membrane of the neuromuscular junction, and 95% of the cases are found to be positive for this antibody. Of all cases with LEMS and SCLC, 64% are positive for SOX1 antibodies [41], and patients are generally admitted with proximal muscle weakness, reduced or absent deep tendon reflexes, and findings of autonomic function impairment. Tumor treatment is known to be a key factor to predict the neurological outcomes. In cases with SCLC, treatment 3,4-diaminopyridine, a potassium channel antagonist, may provide significant recovery, and azathioprine and prednisolone may also be used for the treatment [41, 51, 52].

2.3.5. Opsoclonus-myoclonus

This syndrome is associated with involuntary chaotic conjugated rapid eye movements and myoclonic discharges in the head, neck, face, trunk, and legs as well as potential cerebellar ataxia. In some adult cases, anti-Ri, anti-Hu, anti-amphiphysin, and P/Q-type VGCC antibodies may accompany cancer. Paraneoplastic opsoclonus and myoclonus respond well to treatment. Successful treatment can be achieved by the resection of the underlying tumor and elimination of circulating antibodies such as corticosteroids, intravenous immunoglobulin and plasmapheresis [41, 53].

2.3.6. Chronic gastrointestinal pseudo-obstruction

Chronic gastrointestinal pseudo-obstruction (CGP) is an autonomic neuropathy that is characterized by gastrointestinal dysmotility without a mechanical obstruction, leading to symptoms of abdominal pain, nausea and constipation [41].

While CGP generally occurs in patients with SCLC, it may also be noted in patients with NSCLC. Anti-Hu antibodies are frequently positive. In addition to cancer treatment, agent such as octreotide, prednisone, and azathioprine can be used for the treatment of CGP [41, 54].

2.3.7. Others

Neurological PNS has a variety of clinical manifestations. Other than above-described syndromes, patients may experience subacute sensory neuropathy involving the peripheral nervous system, acute sensory-motor neuropathy (Guillain-Barre Syndrome, brachial neuritis),

neuropathy with vasculitis, myasthenia gravis involving the neuromuscular junction and muscles, acquired myotonia, and acute necrotizing myopathy [41].

2.4. Dermatologic paraneoplastic syndromes

Dermatologic paraneoplastic syndromes are generally seen before patients are diagnosed with cancer. It is not possible to differentiate them from their benign variants in terms of clinical appearance and histopathological findings, although dermatologic PNS that suddenly develop at an atypical localization during the late stages of life and progress rapidly may indicate an accompanying malignancy and it should be investigated [7, 9, 17, 55].

2.4.1. Acanthosis nigricans

Acanthosis nigricans are characterized by skin hyperpigmentation and hyperkeratosis. It is most frequently seen on skin folds such as the axilla, neck, and groins. It usually accompanies lung adenocarcinoma.

The pathogenesis of acanthosis nigricans has not been clarified yet, although one possible etiology is the interactions between increased levels of insulin with insulin-like growth factor receptors and their effect on keratinocytes and dermal fibroblasts [56].

Malignant acanthosis nigricans regresses with the treatment of underlying malignancies, and isotretinoin may be preferred in cases that fail to recover [57].

2.4.2. Polymyositis/dermatomyositis

Polymyositis/dermatomyositis (PM/DM) is a disease characterized by specific skin findings (skin rashes and heliotropic appearance) and inflammatory myopathy accompanying with proximal muscle weakness. Violet-colored edema in periorbital tissues and the eyelids (heliotrophy), periungual telangiectasia, dystrophic changes in the cuticula and macular, violet-colored erythema on the forehead, neck, upper trunk, back, deltoid region, and dorsum of the hand are specific findings of PM/DM [58].

In 15–30% cases of PM/DM, an underlying malignancy is the cause of PNS. Ovarian and breast cancer in women and lung cancer in men are the most common malignancies associated with dermatomyositis [59].

Up to 30% of patients with PM/DM have auto-antibodies against cytoplasmic and nuclear antigens [60].

The treatment of the underlying malignancies can help in the resolution of the findings. Glucocorticoids are the most important medication for the PM/DM treatment, and immunosuppressive agents (azathioprine, cyclophosphamide, etc.) can also be beneficial [44].

LC patients with PM/DM have poor prognosis [61].

2.4.3. Erythema gyratum repens

Erythema gyratum repens (EGR) refers to multiple, erythematosus, serpiginous, concentric-shaped lesions that can grow by almost 1 cm/day [62, 63], but no face, hand or foot involvement is observed.

Rather than benign pathological diseases, EGR generally appears in the presence of malignant diseases and a carcinoma can be detected in more than 80% of patients with EGR. The most common type of malignancy associated with EGR is a SCLC [7, 18].

The pathogenesis of the disease is unknown and its treatment is based primarily on the identification and treatment of the underlying malignancy.

2.4.4. Erythema annulare centrifugum

Erythema annulare centrifugum (EAC) is an eruption characterized by slowly progressing, annular or polycyclic erythematosus lesions.

EAC, caused by benign reasons, is thought to develop due to hypersensitivity reaction. The pathogenesis of a figured erythema developing due to cancer, and thus EAC, is not clearly known, but a suggested hypothesis is that the tumor causes chemical changes in the surrounding tissues, inducing an antigenic status in these tissues, and these antigens lead to inflammation on the skin by causing cross-reactions since they are similar to the skin proteins [63].

EAC regresses with the treatment of the underlying tumor.

2.4.5. Bazex syndrome (acrokeratosis paraneoplastica)

Bazex syndrome is characterized by hyperkeratosis of the acral regions. It appears as erythematosus, papulosquamous plaques on the nose and ears, and less frequently on the fingernails, hands, feet, knees, and elbows. The lesions are generally likened to psoriasis [64, 65]. Benign forms are less frequent than malignant PNS forms.

Its mechanism of development is still unclear, however, it regresses with tumor treatment. Like in most PNS cases, it may appear during LC recurrence.

2.4.6. Tripe palms

It is also known as palmoplantar keratoderma, pachydermatoglyphy, or palmar hyperkeratosis, and is generally seen with LC and 90% of the cases are associated with neoplasm. The most common types of tumors are lung and gastric cancers [41].

2.4.7. Others

Other cutaneous PNS seen in LC patients include paraneoplastic pemphigus, leukocytoclastic vasculitis, multicentric reticulohistiocytosis, sign of leser-trelat, pruritus, and finger clubbing.

2.5. Rheumatologic paraneoplastic syndromes

2.5.1. Hypertrophic osteoarthropathy

Hypertrophic osteoarthropathy (HO) is a syndrome characterized by finger clubbing and periostitis. Secondary HO, which is a PNS, is most frequently seen in LC, particularly in NSCLC [66]. It manifests with a clubbing of the fingers and toes, periostitis of the long bones and polyarthritis in some cases [67].

More than 70% of HO cases are associated with LC, and the incidence rate of HO among LC patients has been found to be 0.7% [41]. Periostitis is a well-known radiographic feature with a generally symmetric distribution, and periosteal reactions involving the long bones are present [67].

While theories have been suggested to explain the mechanisms of HO development, it is still unclear.

In addition to the cancer treatment, nonsteroidal anti-inflammatory drugs, bisphosphonates, and octreotide have been shown to be beneficial for the treatment of HO [41].

2.6. Hematologic paraneoplastic syndromes

Hematologic abnormalities such as anemia, leucocytosis, thrombocytosis, and eosinophilia are common in LC patients. However, not all of these are associated with a PNS. These conditions generally follow an asymptomatic course, and coagulopathies, granulocytosis, anemia, and thrombocytosis can be listed among hematologic PNS [9, 68].

2.6.1. Paraneoplastic coagulopathies

Patients with LC tend to develop thrombosis. Thrombotic risk in lung cancer patients is 20-fold higher than in the general population. Also the risk of thrombosis development in LC is higher than other types of cancer. Several studies have demonstrated that the incidence of cancer diagnosis increases during the first 6 months following venous thromboembolism and venous thrombosis. There are several mechanisms of coagulopathies in patients with LC. The main mechanisms include thrombocytosis, activation of clotting due to injury on the vascular walls, increase in the level and activation of clotting factors, and production of procoagulant factor secondary to tumor hypoxia [18, 29, 69].

Venous thrombosis and hypercoagulability are known as Trousseau syndrome, and while these are mobile in character, long-term anticoagulant treatment should be considered when they are detected.

2.6.2. Paraneoplastic granulocytosis

Granulocytosis, in the absence of infection or leukemia, is relatively common at the time of diagnosis or during the follow-up of patients with LC, and it is seen in 14.5% of patients with LC, and the majority of which are giant-cell lung carcinoma cases [2, 68, 70].

Granulocytosis develops due to the production of paraneoplastic hematopoietic growth factors and certain cytokines (IL-6) [70].

No specific treatment is required other than cancer treatment.

2.6.3. Paraneoplastic anemia

Anemia is seen in several types of cancer. It occurs as a PNS in cancer patients and is a normocytic normochromic anemia. This condition presents with low serum iron levels, normal or elevated ferritin level, normal iron stores, and low serum erythropoietin levels [2].

In LC, anemia as a PNS may also develop depending on autoimmunity, known as autoimmune hemolytic anemia, although antibodies with a specific association are unknown [71].

Anemia generally recovers after the cancer treatment, but patients may be given an erythrocyte suspension in cases of severe anemia.

2.6.4. Paraneoplastic thrombocytosis

Thrombocytosis frequency is 13–32% in LC patients. IL-6 is the cytokine that is known to play a role in the development of paraneoplastic thrombocytosis [41]. It requires no specific treatment.

2.7. Nephrological paraneoplastic syndromes

Nephrological PNS involves a group of disorders that develop as a result of glomerulopathy, which may cause electrolyte imbalance and urea-creatinine elevation, leading eventually to renal failure.

2.7.1. Membranous nephropathy

Membranous nephropathy, developing as a PNS, is most commonly seen in patients with LC, and primarily in patients with NSCLC [72]. Proteinuria leads to the development of hypoproteinemia, resulting in edema in different parts of the body, and acute renal failure and hypertension may also develop in nephrotic syndrome.

The pathophysiology of membranous nephropathy involves the immune response given by tumor-related antigens, and antigen deposits in renal glomeruli have been reported in some patients [25, 73].

2.7.2. Others

Paraneoplastic glomerulopathies seen in LC patients include minimal change disease, IgA nephropathy, focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, crescentic glomerulonephritis, and thrombotic microangiopathies [73].

Effective cancer treatment will be sufficient when paraneoplastic glomerulopathies including membranous nephropathy are detected. However, renal functions should still be monitored and appropriately managed [74].

2.8. Ophthalmologic paraneoplastic syndromes

Ophthalmologic PNS related to the retina or optic nerves can be seen in LC cases, and particularly in SCLC, and retinopathy and optic neuropathy may develop and result in visual dysfunctions. Paraneoplastic syndrome in these cases is caused by the development of immune reaction. The antigens associated with paraneoplastic retinopathy are recoverin and alpha-enolase, whereas collapsin response mediator protein 5 is the antigen associated with paraneoplastic optic neuropathy [75].

Treatment is based primarily on immunosuppressive therapies. However, visual functions may not improve despite immunosuppressive therapy and effective treatment of underlying cancer [76].

2.9. Others

2.9.1. Cachexia

Cachexia is frequently seen in LC patients, and develops as a result of a rather complex process. Roughly, cachexia develops due to the chronic course of systemic inflammation associated with anorexia as well as the loss of muscle and fat mass in cancer patients [77]. While there is no specific treatment for cachexia, the most appropriate approach is to focus on cancer treatment and to provide nutritional support.

2.9.2. Fever

The diagnosis of fever as a PNS is difficult. Several conditions may cause fever in LC patients, including infections (bacterial, viral, parasitic, etc.), drug-induced fever, and autoimmune conditions other than cancer (rheumatoid arthritis, vasculitis, etc.). While the etiology of paraneoplastic fever is not completely understood, it is believed to be mediated by cytokines [78].

In addition to LC treatment, antipyretic medications can be preferred for the treatment.

3. Conclusion

Over the last century, there has been a great deal of progress in the diagnosis and pathogenesis of PNS. PNS is more common in LC patients and its frequency is higher in SCLC than in other types of LC.

PNS may involve almost all systems and may appear before or after the diagnosis of cancer. The diagnosis of the lesions that appear before LC diagnosis can significantly affect the outcomes by allowing the early identification of LC and changing its prognosis through timely treatment. Similarly, recurrences and remissions of PNS provide important clues during cancer follow-up.

A clear understanding of the mechanisms leading to PNS development in LC patients and improvements in the diagnostic and treatment methods will significantly provide positive improvements in the cancer treatment.

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Pleural Effusions in Lung Cancer: Detection and Treatment

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Additional information is available at the end of the chapter

Abstract

In all cell types of lung cancer, pleural effusion is a possible complication of disease. Paramalignant pleural effusions [PMPE] are not a consequence of malignant disease spreading to pleura. The probability that an effusion is paramalignant is higher if the effusion is a transudative or parapneumonic effusion. Differentiating between para-malignant and malignant effusions has both therapeutic and prognostic significance. MPEs are a sign of metastatic dissemination of neoplastic disease. In pleural fluid or tissue, there are malignant cells. In PMPE, lung cancer had been previously diagnosed. Bronchoopstruction, atelectasis, infection, pulmonary emboli, air therapy, and helio-therapy result in effusion development. PMPEs equally appear in all pathohistological types of lung cancer, as MPEs are the most common in lung adenocarcinoma. Also, there are biochemical properties of PMPE and MPE. Therapeutic procedures depend on the presence of respiratory distress, biochemical properties of pleural fluid, type of primary tumour, and expected response to the therapy.

Keywords: paramalignant, malignant, effusion, thoracocentesis, pleurodesis

1. Introduction

Over 175,000 MPE [1] are diagnosed yearly in USA and 50,000 [2] in the UK. In 75% of cases, MPE are a consequence of metastatic dissemination of lung or breast cancer [3]. Pleural effusion in lung cancer is a complication of terminal or preterminal stage of disease. Lung cancer disturbs one or more mechanisms of normal fluid flow, which is followed by inevitable accumulation of fluid in pleural space. Pleural effusion is not always a sign of cancer metastasising, but it is evident that in most cases it is related to the primary disease.

Pleural effusion is a possible complication of disease in all cell types of lung cancer [4, 5]. At the first presentation, around 15% of patients with lung cancer have a pleural effusion [5]. For the duration of disease, approximately 50% of patients develop a pleural effusion. Depending on the presence of malignant cells in pleural fluid, pleural effusions are divided into two groups: paramalignant pleural effusions [PMPE] and malignant pleural effusions [MPE] [3]. Distinguishing between PME and MPE can be challenging. Analytical limitations do not allow the use of a single method to pursue the diagnosis and also can expose the patients to invasive procedures. PMPE are not a consequence of malignant disease spreading to the pleura. The probability that an effusion is paramalignant is higher if the effusion is a transudative or parapneumonic effusion. Differentiation between PMPE and MPE is important, so that appropriate decisions about treatment modalities can be made, and also due to different prognosis of the two conditions.

2. Clinical features of pleural effusion

In patients with lung cancer and pleural effusion, there is a mild to medium symptomatology of respiratory distress. Clinical history usually points to the diagnosis of lung, breast, ovarian cancer or lymphoma. At the time of diagnosis of malignant pleural effusion, 23% of patients are asymptomatic [4]. Pleural effusion followed by pleural pain indicates the inflammation of the parietal pleura. Dull pain in the chest wall stirs suspicion of pleural malignancy [6]. Nevertheless, pleuritic or dull pain in the chest wall indicates a distortion of the parietal pleura and a high probability of an exudative effusion developing. As a rule, pain is a consequence of pleural disease. Localisation of pain is correlated to the area of pleura that is affected (parietal pleura is innervated by intercostal nerves). Occasionally, pleuritic pain spreads into upper parts of stomach [intercostal nerves innervate the abdomen as well]. Exception in pain localisation and spreading of the pain are noted when the central parts of diaphragmatic pleura are affected by the disease. These parts of the pleura are innervated by phrenic nerves and consequently the pain localises to the ipsilateral shoulder. In over 70% of patients with MPE intrathoracic pain is a symptom of the disease [7]. Non-productive cough can also be a symptom of a pleural effusion. Mechanism of genesis of the cough is not clear and is probably related to pleural inflammation. Alternatively, compression of lungs and bronchial wall by fluid might stimulate the cough reflex [8]. Cough is present in over 50% of patients. A common symptom of pleural effusion is dyspnoea. Dyspnoea is present in around 70% of patients with MPE [7]. The severity of dyspnoea is often not proportional to the size of pleural effusion [4]. Dyspnoea is usually present in diaphragmatic dysfunction. In an inverted diaphragm, dyspnoea is disproportional to the size of the effusion. Mulvey [9] classified hemi-diaphragmatic alterations seen on chest radiographs and fluoroscopy into three groups. The three groups are: normal function of the hemidiaphragm, fixed hemidiaphragm, and hemidiaphragm with paradoxical movement. Patients with normal functioning diaphragm are usually asymptomatic, even with a large pleural effusion. The second group of patients are the patients with a fixed hemidiaphragm. Immobile diaphragm disables sufficient ventilation of the lungs. In the third patient group, hemidiaphragm exhibits paradoxical movement which results in severe dyspnoea. Paradoxical movement of the right hemidiaphragm is rarely seen, probably due to proximity of the liver. The severity of respiratory

insufficiency depends on the size of the effusion and previous lung function. Pleural effusion reduces the thoracic space and lung volumes or in turn the thoracic cavity enlarges as the ipsilateral hemidiaphragm descends. Therefore, fluid in the pleural space causes restrictive ventilator defect. Small to moderate pleural effusions cause dislocation rather than lung compression and they have a little consequential effect on the lung function [6]. In massive pleural effusions, the most common symptoms are the ones that are a direct consequence of lung function compromise. Improvement of lung function after therapeutic thoracocentesis is less than expected [10]. Explanation of inadequate improvement of lung function probably lays in the fact that usually, besides pleural effusion, there are also changes in the lung parenchyma. In massive pleural effusions, the mechanism of dyspnoea is closely related to the reduction of chest wall compliance, counter lateral mediastinum movement, and loss of the ipsilateral lung volume with additional action of neurogenic factors of the pulmonary parenchyma [11].

In 26 patients, spirometry was performed before thoracocentesis and 24 hours after thoracocentesis. Average amount of evacuated fluid was 1740 ml [12]. After thoracocentesis, vital capacity increased for 410 ± 390 ml. Estenne et al. [13] examined respiratory mechanics in nine patients, before and 2 hours after evacuation of 600–2750 ml (average amount = 1818 ml) of pleural fluid. Before thoracocentesis, forced vital capacity (FVC) was between 22 and 51% of predicted values. After thoracocentesis, the average value of FVC has increased only by 300–460 ml. Estenne interprets reduction in dyspnoea by reduction in the size of the thoracic volume. Reduction of the thoracic cavum enables inspiratory muscles to function in much more favourable length-tension relationship. Before thoracocentesis, maximum inspiratory pressure at TLC (total lung capacity) was −16 cm H_2O, and after thoracentesis, the pressure fell to −25 cm H_2O. Explanation of these phenomena probably lays in the fact that hemidiaphragm is released from the pressure of pleural fluid. In pleural effusion, PaO_2 is usually low, as alveolar-arterial gradient is increased.

Patients with pleural effusion complain of intolerance to exertion. Exertional intolerance was examined in 24 patients, before and after thoracocentesis. There were no significant changes in hypoxia and hypercapnia level. A number of patients had malignant effusions and it is possible that the given results are the consequence of primary disease rather than lung function being compromised by pleural effusion [14].

Systemic symptoms are a consequence of cancer development. The most common systemic symptoms are weight loss, general weakness, haemoptysis, fever, cyanosis, and dysphagia [4]. Over 40% of patients had systemic symptoms, general weakness, and loss of weight. Eighteen percent of patients had fevers, and 9% of patients had haemoptysis [13]. Similar data were found by Chernow, Sahn [4] and Baburao and assoc. [15]. The patients with MPE had chest pain in 32% cases, compared to 11% of patients with benign effusions, while the patients with benign effusions were more commonly found to have pleuritic pain [51 versus 24%]. Fever was more common in the patients with benign effusion (73 versus 37%) [8].

Massive pleural effusion can compromise cardiac function. It has been proven that massive pleural effusion can lead to the right ventricular diastolic collapse with consequential reduction of cardiac output. In artificial bilateral pleural effusions, right ventricular diastolic collapse appears at the pressure of about 4 mmHg [16]. This value of pleural pressure is also seen with the patients with massive pleural effusion [17]. Arterial-blood gases usually have clinically acceptable values.

On examining the patient, the absence of the pectoral fremitus, shortened and dull percussion sound, and weakened respiratory sounds are usually found. By auscultation above the fluid level, the change of breathing pattern (area of the compromised lung), that is, aegophony is heard. Conversing through auscultation of the affected side, increased resonance of voice can be heard. Experienced doctors think that dullness to percussion and aegophony are two most common findings that are present in over 90% of patients with pleural effusion. Above the area of dullness to percussion, humid sounds are heard ausculatorily [18]. Contralateral displacement of the trachea could be found in massive effusion.

3. Paramalignant pleural effusion

The main characteristic of PMPE is that the lung cancer had been previously diagnosed, and that malignant cells had not been identified in the effusion either cytologically or pathohistologically. These effusions are not a sign of malignant disease spreading to the pleura [3].

PMPEs are a direct consequence of local or systemic effect of tumour. Pulmonary infection distally from partial or complete obstruction of bronchi could be a cause of parapneumonic effusions. Obstruction of main or lobar bronchi by neoplasm leads to consequential atelectasis of the corresponding part of the lung. In order to compensate for the lost volume, the remainder of the lung must additionally expand or the hemithorax must be contracted. This sequence of events results in lower intrapleural pressure. Low intrapleural pressure is an additional factor of more intensive fluid accumulation.

Furthermore, the incidence of pulmonary emboli in malignant disease is not negligible, and pulmonary embolus can also be one of the causes for pleural effusion development. Additionally, certain chemotherapeutic protocols are a cause of increased retention of fluid in pleural space. Lymphatic obstruction is a common property of lung cancer and lymphoma and at the same time is a possible complication of radiotherapy which is a contributing factor of fluid accumulation in pleural space.

A considerable number of patients with malignant disease are malnurtured. Hypoproteinemia can in rare cases lead to transudative pleural effusion. In malignant disease, metastatic changes on the pericardium can be found. Pericardial effusion leads to the increase of hydrostatic pressure in systemic and pulmonary circulation which leads to transudative pleural effusion development [19].

In around 5% of patients with lung cancer, PMPEs are diagnosed. Frequency of PMPE in squamous cell carcinoma is 4.5%, in adenocarcinoma 6.2% and in SCLC 6.5%, so they are equally present in all pathohistological types of lung cancer [7, 21].

In 67% of patients, PMPEs are moderate or massive. They are mostly serous or cloudy (60%), and haemorrhagic effusions represent 21.3% of all PMPEs [7, 20].

3.1. Pleural fluid analysis

Compared to the MPE ratio of LDH—E/S (effusion/serum) is higher in PMPE, but not significantly higher. This can be interpreted with the help of the fact that in PMPE, parapneumonic

effusion (inflammation is followed by high values of LDH in effusion) is diagnosed in 18.3% of patients. Significant differences in other biochemical characteristics (glucose, proteins, and cholesterol) were not found either [7].

Compared to MPE, total number of cells, eosinophils, and PMN [polymorphonuclear cells] is higher in the group of patients with PMPE, but not significantly. Lymphocytes and PMN dominate in the differential picture in PMPE. Percentage of PMN is usually below 25%, but cases when they dominate are not rare, for example, in secondary pleural inflammation. In PMPE, eosinophils are found in 51% of patients diagnosed with PMPE. The percentage range of eosinophils in pleural fluid ranged from 1 to 24% [7].

3.2. Management of paramalignant pleural effusion

PMPEs are not an absolute contraindication for operative treatment. It is a therapeutic challenge to establish the optimal therapeutic protocol for the patients who are diagnosed with lung cancer and ipsilateral pleural effusion in which no malignant cells are found cytologically. Rodriguez-Panadero [21] performed thoracoscopy on 21 patients with lung cancer and paramalignant effusion. After thoracoscopy, thoracotomy was indicated in five patients. Invasion of mediastinal lymph nodes was found in all five patients. In another study, five patients out of 73 with PMPE have survived long term after surgery [22]. It can be concluded that in the patients with PMPE, thoracoscopy and CT of the thorax should be firstly done for the evaluation of mediastinal lymph nodes. In the case of enlarged lymph nodes, mediastinoscopy is indicated. In the patients with negative thoracoscopy, even if lymphatics are not enlarged, explorative thoracotomy is recommended. Before resection, lavage of pleural space with cytological analysis of the obtained material should be performed. One study that encompassed more than 1200 patients found that in patients with lung cancer without an effusion undergoing surgical resection with curative intent, 5.3% of patients had positive cytological findings of the pleural fluid lavage at the time of thoracotomy before surgical resection took place [23]. In PMPE, most commonly performed therapeutic procedures are thoracocentesis (51%) and pleurodesis (39%). Thoracotomy was done in two (6%) patients [7].

4. Malignant pleural effusion

Malignancy is the most common cause of massive pleural effusions opacifying the entire hemithorax and of large pleural effusions opacifying two thirds of a hemithorax. MPE is an end-stage sign of malignant disease. Sometimes MPE can be the first clinical sign of a tumour, given that often lung cancer are not detected in the beginning of disease development. In a retrospective series of 766 patients, carcinoma was the cause of 55% of massive and large pleural effusions [24]; other causes included tuberculosis effusion and pleural empyema.

In older age groups (>60 years old), malignant pleural effusions are the most common cause of exudative effusions and they are often the first clinical manifestation of disease (**Figure 1**) [3, 5]. Diagnosis of malignant effusion is established by the identification of malignant cells in effusion or pleural tissue. Pathohistological and/or cytological investigations are not of credible diagnostic significance until malignant cells are defined.
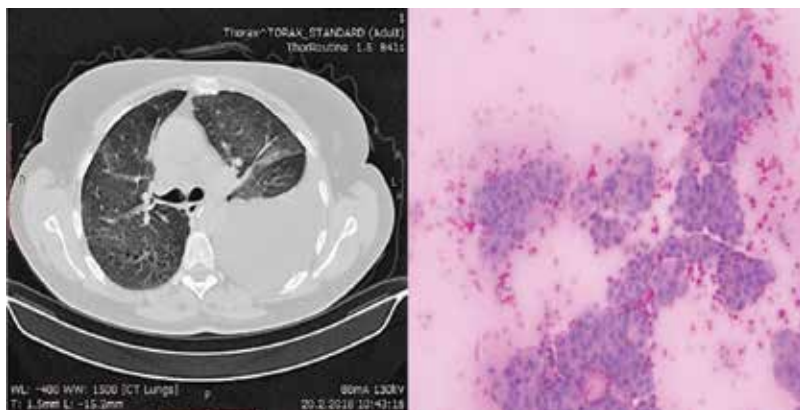
Figure 1. Pleural fluid sample containing cells of adenocarcinoma. N.B.—Origin of the primary tumour is unknown.

In over a half of MPE in men, the effusion is caused by lung cancer. MPEs appear in all cell types of lung cancer [3]. Incidence of MPE in lung adenocarcinoma is significantly higher in comparison to other pathohistological types of cancer. Thirty one percent of patients (out of 96) with lung adenocarcinoma developed a MPE, while 8.6% of patients (out of 404) with squamous cell carcinoma and between 7 and 10% with patients with small-cell carcinoma developed a MPE [7, 25–27].

The next most common causatives of malignant effusions are lymphoma and leukaemias (20%). Seven percent of MPE are a result of spread of gastrointestinal system cancer, 6% of genitourinary system and 11% of tumour are of unknown primary source [28]. Incidence of MPE in regards to primary tumour localisation is given in **Table 1**.

In women, around 40% of MPE is a direct consequence of spread of breast cancer. In about 50% of patients with breast cancer, pleural effusion is developed during evolution of disease [29]. Twenty percent of effusions are the consequence of genital tract tumour spread, 15% of lung malignoma, 8% of lymphoma or leukaemia, 3% of melanoma, and 9% of tumours of unknown primary source.

Primary tumour localisation	Incidence %
Breast	26–49
Lung	10–24
Lymphoma	Up to 24
Non-Hodgkin lymphoma	13–24
Ovary	6–17
Hodgkin's disease	Up to 13

Table 1. Causes of malignant pleural effusions.

4.1. Pathophysiology of malignant pleural effusions

Malignant tumours can lead to pleural effusion development either by direct or indirect spread of the disease. In post-mortem studies, malignant disease of the pleura without an effusion was found in 40% [30] to 45% [6] of cases. Pleural effusions in malignancy most likely develop due to increased entry of fluid into pleural space and decreased evacuation of liquid from the pleural space. Increased transport of liquid into pleural space occurs due to increased permeability of the pleural vessels by direct invasion of the tumour cells, vasoactive and inflammatory cytokines. Increased permeability of pleural vessels can also be caused by injury, infection, pulmonary embolus, pulmonary infarction that causes movement of liquid from lung to pleural space by increased hydrostatic forces caused by venous obstruction [31].

Pleural liquid and protein are largely resorbed by the lymphatic system of the parietal pleura. The exit of fluid from pleural space could be decreased by several mechanisms that reduce lymphatic drainage. Lymphatic obstruction at any point, from the stomata of parietal pleura to mediastinal lymph nodes is a dominant cause of increased accumulation of pleural fluid [3, 20]. Insufficiency of lymphatic drainage appears for two reasons. The first one is that the transport of liquid from pleural space through stomata and lymphatic vessels of parietal pleura is disabled because of the presence of metastases and other reason, as the lymphatic vessels of parietal pleura are mostly drained by mediastinal lymph nodes. So, neoplastically changed mediastinal lymph nodes decrease the clearance of pleural cavum thus contributing to additional accumulation of fluid [32]. Obstruction of thoracal ductus by malignant tumour can also be a cause of pleural effusion development. In these cases, a chylothorax develops. Chylothorax is the most common complication of mediastinal tumours, mostly lymphoma (in 50%). Furthermore, chylothorax is a complication of surgery (20%) and trauma to the thorax (<5%) [33].

In atelectasis pleural pressure is lower due to bronchial obstruction and therefore the exit of fluid from pleural space is reduced. In superior vena cava syndrome, pleural liquid drainage is reduced due to elevated central venous pressure.

Cellular and molecular mechanisms of localisation of pleural metastases are still mostly unclear. From visceral pleura malignant cells spread to parietal pleura, where they multiply [20]. Pleural metastases are also found on visceral and parietal pleura, while isolated metastases of parietal pleura have never been identified. From visceral pleura, malignant cells spread on parietal pleura [3]. In the process of pleural metastase development, several steps are necessary. First, malignant cells must leave the primary tumour. For this step, deregulation of cellular adhesion is necessary. Deregulation of cellular adhesion depends on the changes in the extracellular matrix and the change of expression degree of integrin with simultaneous increase of cell motility. By leaving the primary tumour, tumour cells via vascular or lymphatic structures (they can cause different haemodynamic and immunological changes in these structures) find their way towards distant organs or lymph nodes. With the help of video microscopy, it has been shown that a great number of circulating tumour cells (80%) stay alive even up to 3 days after their entry, both into circulation and/or extravascular space. Only small subgroups of cells (0.07%) can form metastases [34].

Control of extravascular cell growth is crucial in forming of metastases. Formation of pleural metastases is probably mediated by the interaction of mesothelial and neoplastic cells. Tumour activated mediators (VEGF, ligand for CC-sequence of chemokines CCL2 and TNF) stimulate the accumulation of inflammatory cells [35, 36]. IL-5 stimulates the development of MPE, accumulation of Eo as well as myeloid suppressor tumour-activating cells [37]. On the other hand, tumour cells activate proinflammatory and proangiogenic transcription programs controlled by nuclear transcription factor (NF)-kB [38] and signal transduction of transcription activator (STAT)3 [39]. Tumour necrosis factor (TNF), interleukin 6([IL-6), and osteopontin (OPN) participate in positive reverse coupling (povratna sprega) regulating the activation of tumour NF-kB/STAT3. The end result of these events is the formation of MPE [35]. Furthermore, stimulated mesothelial cells secrete different factors, such as chemoattractants, chemokines, and platelet-derived growth factor (PDGF). These products facilitate the appearance of metastases. Simultaneously, the production of adhesion molecules is increased, such as intracellular adhesive molecules [ICAM-1] and vascular molecules of cell adhesion (VCAM-1) which, in vitro, in contact with cancer cells secrete metalloproteinase [40].

And finally, continuous growth of metastatic foci depends on angiogenesis. Vascular endothelial growth factor (VEGF) stimulates proliferation and migration of endothelial cells. At the same time, invasion of pleural space by malignant cells and VEGF expression from tumour cells is necessary for the formation of pleural liquid [41]. Angiogenesis is the primary process that disables the growth and progression of tumour [42]. Yano et al. [41] used human cells of pulmonary adenocarcinoma and squamous cell cancer, with different invasive properties and different levels of VEGF expression, and they have proven that invasion of pleural space by malignant cells and VEGF expression from tumour cells is necessary for the formation of pleural fluid. VEGF is one of the most powerful known chemokines that directly affect the increase of vascular permeability [42]. In comparison to tuberculosis effusions and transudative effusions, the concentration of VEGF is significantly higher in malignant effusion [42, 43]. The average value of VEGF in malignant pleural effusion is significantly higher than in pleural effusions that are a consequence of congestive cardiac failure [41].

Malignant effusion in comparison to benign incidence of CD4+ cells is significantly increased [44]. Accumulation of immunosuppression and protumour CD4+ lymphocytes contributes to weakening of the immune response and simultaneous growth of tumour cells [45].

On development of pleural metastases, tumour cells disseminate onto the mesothelial surface or malignant cells penetrate the subserosa. When the mesothelium is encompassed by the tumour, 'excess' of tumour cells appears in the pleural fluid. However, when only submesothelium is infiltrated by the tumour, there is a small number of malignant cells in pleural liquid. In these cases, malignant cells are rarely identified in pleural fluid, that is, thoracocentesis does not have any diagnostic significance [46, 47]. Infiltration of pleura by tumour results in reactive changes of mesothelium, that is, fibrosis. In advanced stage of disease, disposal of collagen into submesothelial pleural tissue is increased, which is at least a part of the cause of low values of pH and glucose in the effusion.

Another, and less likely mechanism of metastases development is by direct invasion of pleura by tumour, whether it is lung or breast cancer. In some cases, spread of tumour to the pleura is evident, and in spite of this, the effusion does not develop. For example, effusion rarely

appears when pleura is invaded by sarcoma. One of the properties of sarcoma is the absence of lymphogenic metastases [20].

Bilateral metastases in lung cancer are usually indirect evidence of the primary neoplasm spreading to the liver and of subsequent disease dissemination to the lungs. However, if the lungs are not the primary source of carcinoma, this implies that the changes on pleura are a tertiary consequence of metastasis spreading from the liver [4, 19].

4.2. Pleural fluid analysis

Pleural effusions in lung cancer are usually classified as exudates [4, 48]. In 5–10% of cases, the pleural effusion is a transudate [4, 54]. Transudative effusions are more likely when the lymphatic drainage is obstructed by the tumour and in atelectasis caused by bronchial obstruction or congestive cardiac failure [31]. Malignant cells can be found in transudative effusions as well. Macroscopic MPEs can be serous, serosanguinous, and haemorrhagic. Erythrocyte count in pleural fluid is often between 30,000 and 50,000/µl [48]. Erythrocyte count over 100,000/µl, in the absence of trauma, indicates a probable diagnosis of malignant disease. Direct invasion of blood vessels, occlusion of venules, increased permeability of capillaries due to vasoactive chemokines and cytokines, occlusion of venules usually result in a bloody, malignant pleural effusion.

In more than half of malignant effusions, lymphocytes are present in 50–70% range, but are typically present in lesser amount than they occur with tuberculosis pleurisy (usually ≥80%) [12, 49]. In TBCPE, the number of lymphocytes was $153,696 \times 10^4$ versus $95,414 \times 10^4$/ml in MPE [7]. In order to differentiate between tuberculosis and malignant effusions, determining adenosine deaminase (ADA) presence in the effusion is of significance. The level of ADA greater than 70 U/l in effusion indicates to tuberculosis aetiology, while the ADA level below 40 U/l excludes the tuberculosis aetiology [50, 51]. Although the cause of lymphocytosis is unclear, the lymphocytes that are most prevalent are T lymphocytes that have a role in local defence systems against tumour invasion of the pleural cavity. In vitro, lymphocytes of malignant pleural effusions spontaneously secrete lymphokines [52]. Mesothelial cell percentage in malignant effusion varies ranging from few percent to a large percentage of total cells. Mesothelial cell abundance occurs early in the course of infiltration of the pleura, well before pleural fibrosis and marked tumour infiltration. Fewer mesothelial cells are seen in advanced stages of pleural metastases due to pleural fibrosis.

The percentage of PMN is usually below 25%, even though often PMN can dominate the percentage, as in secondary inflammation of pleura. Eosinophils are present in up to 36% of malignant effusions [7]. The percentage in which eosinophils are a significant finding in pleural liquid is considered to be higher than 10%. In MPE, eosinophil percentage ranges from 1 to 52% [7]. Eosinophilic pleural effusion is the most common in malignant disease (34, 8%) [24], which is then followed by infections (19, 2%), effusions of unknown aetiology (14, 1%), post-traumatic effusions (8, 9%), and effusions (23%) in other diseases.

Usually, eosinophilia is related to the presence of blood or air in pleural space. Eosinophilic effusions reappear even after repeated thoracocentesis [53]. Good correlation was found between IL-5 levels and the number of eosinophils in pleural fluid [54]. In MPE, compared to

other cells, malignant cells are rarely found. Significant concentrations of proteins are found in pleural fluid. Nevertheless, the total amount of protein transported from pleural space is lesser than the amount of protein transported in tuberculosis effusions, effusions in pulmonary embolism, and congestive cardiac failure.

Chronic pleural effusions (CPE) are very rarely found to be transudates. Characteristic biochemical property of CPEs is that they have low pH and low glucose concentration, while they have a large protein concentration. The ratio of pleural protein to serum protein can found to be low, and nevertheless, the effusion would still be classified as an exudate. If in exudative effusion only the LDH criterium is positive, this points to the diagnosis of malignancy [48, 55, 56].

In one third of MPE, pH is less than 7.30 (ranging from 6.95 to 7.29) [57]. Effusions with low pH value have a high concentration of glucose (<60 mg/dl) and pO_2, and additionally a high concentration of lactate and pCO_2. Glucose concentration can be rarely reduced to the value of 5 mg/dl and it usually ranges from 30 to 55 mg/dl [55]. Malignant effusions with low values of pH and glucose characteristically have a longer evolution of up to several months and they usually follow the fast tumour and pleural fibrosis [54]. Pathologically altered pleura reduce glucose entry into the pleural space and at the same time disable the transport of metabolic products. The end result of this disorder is local acidosis [56]. Low level of LDH in effusion indicates a higher possibility of negative pathohistological results. In effusions with high LDH level, the percentage of positive biopsies is higher [58].

Pleural fluid amylase concentration is found to be elevated in 10% of patients diagnosed with a MPE. However, the origin of amylase in pleural fluid is not pancreatic but is found to be salivary instead. In a series of consecutive effusions, a very high amylase level in malignant pleural fluid (>600 IU/L) was found to be a poor prognostic factor [59].

4.3. Tumour markers in pleural liquid

Biological markers of malignancy are yet to be identified, but may be identified in the future as the molecular biology of cancer is better understood. Pleural fluid biomarkers could potentially assist the cytological diagnosis. Unfortunately, biomarkers have been found to have indeterminate specificity and sensitivity which has led to overlap between malignant and benign conditions. An approach that has been used is to combine the tumour markers to improve the diagnostic yield. However, the diagnosis of malignancy can also be reached by using the clinical characteristics—duration of symptoms for more than a month, absence of fever, CT thorax findings of malignancy, and serosanguinous fluid [60].

4.3.1. Carcinoembryonic antigen

It has been concluded in several reports that determining the CEA level in pleural fluid is useful in establishing the diagnosis of malignant pleural effusion [61, 62]. CEA level below 10 ng/ml is usually found in lymphoma, sarcoma, and mesothelioma. CEA higher than 10 ng/ml could indicate a malignant disease; however, it does not have diagnostic significance, and routine measuring of CEA is not recommended.

4.3.2. Carbohydrate antigen

It is known that three different carbohydrate antigens (Ca 15-3, CA 19-9, and CA 72-4) are present in malignant disease. These antigens have been studied in order to differentiate malignant and benign effusions [63]. Comparing the levels of these three antigens in malignant and benign effusions, there is a significant overlap of the obtained results. Hence, this test is not sensitive enough and it is not used in routine diagnostics.

4.3.3. Sialyl stage-specific antigen-1

Sialyi stage-specific antigen-1 is a carbohydrate antigen present in malignant disease. Patients with positive cytological findings of pleural fluid to adenocarcinoma cells have higher values of this antigen in effusion in comparison to effusions of other aetiologies [64]. However, simultaneously, significant overlap of value levels of this marker was recorded, and therefore sialyl stage-specific antigen-1 in effusions of unclear aetiology does not bare a diagnostic significance.

4.3.4. Cytokeratin-19 fragments

Cytokeratin 19 (CYFRA 21-1) is the main component of cytoskeletal filaments of epithelial cells. It is significantly increased in malignant diseases. The level of CYFRA-21 higher than 100 ng/ml is present in around 60% of patients with carcinoma and mesothelioma [65]. A significant overlap has been found in CYFRE levels in both malignant and benign effusions, and therefore this test is not recommended in routine work.

4.3.5. Enolase

Enolase is a glycolytic enzyme present in extracts of neuroendocrine tumours. In pleural effusions in small cell lung cancer, higher levels of enolase are found when compared to pleural effusions in non-small cell lung cancer and benign effusions [66], but as there is a significant overlap in measured enolase values establishing its presence has no diagnostic significance.

4.3.6. Squamous cell carcinoma antigens

Squamous cell carcinoma antigen (SCC) has been used as a serum marker for squamous cell carcinoma. In the greatest number of patients with pleural effusion in malignant disease, a low level of this marker was found. This marker was positive in 7 out of 11 patients (64%) with squamous cell carcinoma. However, SCC values are very high in some benign effusions as well; thus, SCC is not used in squamous cell carcinoma diagnostics [66].

4.3.7. Oncogenes

Oncogenes are closely related to the development of malignancy, and one of the hypotheses has suggested that in the pleural fluid of patients with pleural malignancy cells containing oncogenes are present. In 11 (34%) of malignant effusions, protein p53 has been detected, and should be noted that it has not been found in any non-malignant effusion [67].

There is no significant difference in CMYC oncogene expression between malignant and benign effusions [68]. CHARAS oncogene has been detected in 21 out of 24 malignant effusions, but it has also been found in 6 out of 16 benign effusions (37%), and therefore its diagnostic utility is limited [69].

4.3.8. Hyaluronic acid

Pleural fluid of patients with mesothelioma is sometimes extremely viscous. Increased viscosity of liquid is a consequence of hyaluronate, that is, hyaluronic acid presence. Hyaluronic acid level higher than 1 mg/ml indicates mesothelioma diagnosis. In no other aetiology of pleural effusion, hyaluronate level higher than 0.8 mg/ml was found. Sensitivity of this test for malignant mesothelioma is 56% [70]. Average values of hyaluronic acid in mesothelioma are comparable to values of hyaluronic acid present in pleural effusions in adenocarcinoma [71].

4.3.9. Lectins binding

Lectins are a class of glycoproteins of non-immune origin which bind specifically to the carbohydrate group in different biological products. Lectin binds much easier to adenocarcinoma cells compared to reactive mesothelial cells or mesothelioma cells [72]. These authors could not find significant differences in lectin binding between mesothelioma cells and reactive mesothelial cells.

4.3.10. Flow cytometry

Flow cytometry is a method of quick measuring of nuclear DNA. It has been postulated that this method would enable differentiation of benign from malignant cells, taking into account that malignant cells have numerous chromosomal abnormalities (aneuploidy) and consequentially abnormal DNA content (DNA aneuploidy). However, aneuploidy is present in benign effusions as well [73]. Flow cytometry is used in the identification of superficial lymphocyte markers and it has found the use in diagnosis of lymphoma [68].

4.3.11. Chromosomal analysis

Malignant cells have a higher number of chromosomes with structural abnormalities, such as translocation, ascension, dissension, discention, inversion, and isochromosomy [70]. The place of chromosome analysis in routine examination of pleural effusions remains to be established.

4.3.12. Association of mRNA expression in metastatic malignant effusions

Alternative diagnostic methods are still needed to assist in the diagnosis of pleural effusions. The supernatant of samples of pleural effusion might contain useful information such as nucleic acids and proteins. MicroRNAs that are circulating and cell free have been identified as potential biomarkers of cancer. MicroRNAs are heavily involved in processes of development, cell survival, carcinogenesis, and apoptosis. Hence, it is likely that they play a considerable role in modulating sensitivity and resistance to anticancer medications [74].

However, potential applications and the existence of cell-free microRNA in pleural effusion samples are uncertain. MicroRNA (miRNA) is a group of short RNAs that regulate expression of proteins post-transcriptionally by binding to the 3'UTRs target mRNAs. As MicroRNAs are involved in cancer development, the expression of a specific miRNA profile may suggest the disease status, prognosis, and response to chemotherapy agents.

Wang et al. [75] have used real-time quantitative PCR to analyse related gene expression in 46 patients with malignant effusion. Data were prospectively collected from gastric cancer, non-small cell lung cancer, and gynaecological cancer patients. Cancer cells that are viable and obtained from malignant effusions are tested for sensitivity to docetaxel and cisplatin using ATP-TCA assay. The authors have concluded that BRCA1 (breast cancer susceptibility gene 1) and ERCC1 (excision repair cross-complementing group 1) miRNA expression levels are in correlation with in vitro chemosensitivity to docetaxel and/or cisplatin in malignant effusions of gastric cancer and non-small cell lung cancer patients. Additionally, combining ERCC1 and BRCA1 may produce better results predicting the sensitivity to cisplatin than when only a single agent is considered.

4.4. Prognosis of malignant pleural effusion

By diagnosing a malignant effusion, prognostic information is obtained simultaneously [57]. Mean survival time of patients with lung cancer and MPE is between 3 and 4 months [76]. Multivariable analysis has demonstrated that shorter survival time was found in patients with a high level of serum CRP, low values of albumins, serum proteins, distant metastases and those patients who did not have chemotherapy [77]. Disease progression and poor prognosis can be related to the immunosuppressant effect of tumour and functional damage of the immune system.

In non-small cell carcinoma, mean survival time in patients with stage IIIb, IIIb with pleural effusion and stage IV was 15.3, 7.7, and 5.5 months, respectively [78].

From the time malignant, effusion is diagnosed, patients with lung, stomach, and ovarian cancer survive for only several months, while survival time in patients with breast cancer is longer—several months or years, depending on the response to chemotherapy [4, 55]. Survival time of the patients with lymphomatoid effusion is between the survival time of patients with breast cancer and cancers of different organs.

Prognosis of malignant effusion depends on the stage of the disease and contributing factors (**Table 2**) [55], which is of crucial importance for choosing the treatment modality. LDH criterium and pleural fluid pH in malignant effusions are important prognostic indicators. Poor prognosis is indicated by high LDH effusion/serum ratio, high level of LDH in effusion, and low pH of pleural fluid [55]. When pH and glucose levels are found to be low in a malignant pleural effusion (below 7.30 and 60 mg/dl, respectively), the survival time is found to be less for an average of 2 months when compared to those patients who have normal values of glucose and pH-average of 10-month survival time [76].

The only true predictive marker of mortality might be the performance status at the time of diagnosis. A Karnofsky score of more than 70 is associated with a median survival time of 13.2 months, while a Karnofsky score of less than 30 is associated with a median survival time of 1.1 months.

Tumour related	Non-tumour related
Extensive invasion of mediastinal lymph nodes	Tuberculosis or fungal infection
Parapneumonic effusion (obstructive pneumonia)	Immunological disorders: rheumatic, allergic, etc.
Pericardial effusion as a consequence of malignant disease	Pulmonary embolism
Previous mediastinal radiotherapy	Congestive cardiac failure
Malignant ascites	Parapneumonic effusion as a consequence of an unrelated pneumonia
Pulmonary embolus	Organ failure that is not a consequence of cancer or cancer treatment

Table 2. Factors contributing to the spread of pleural effusion.

4.5. Imaging

4.5.1. Chest radiographs

For documenting the presence of pleural fluid in pleural space, the most practical diagnostic test is conventional chest radiography [79]. Pathological changes of the pleura are commonly easily diagnosed by appropriate radiological modalities.

The distribution of fluid in pleural space is completely dependent on the laws of gravity. Fluid is firstly accumulated in basal parts of the hemithorax, that is, between the lower surface of the lung and diaphragm. When a large amount of fluid accumulates, the fluid starts spreading to anterior, posterior, and lateral costophrenic sinuses. The thickness of the fluid is greater laterally. The line of the border discretely fades on moving medially and ends in the mediastinum with a meniscus shaped line. On lateral radiographs, upper border of pleural fluid is semicircular, that is, the upper border is higher both anteriorly and posteriorly, while it gently descends in the middle part. A small amount of fluid seen on the chest radiograph is not indicative of a pleural effusion, as the diaphragmatic configuration is unchanged. During accumulation of a larger amount of pleural fluid, costophrenic angles are the first to be filled. Costophrenic angles are filled only after the quantity of fluid in sub-pulmonary space exceeds 175 ml s.

'Middle lobe step sign' can be often seen on a lateral view. The 'middle lobe step sign' is explained by the fact that the fluid firstly accumulates in the lower lobe, as it is the lowest of the pulmonary lobes. The middle lobe remains unchanged and its volume is preserved. Pleural fluid is most commonly evident in the posterior parts of the chest.

In massive pleural effusion, the sheer weight of the fluid can be a cause of inverted diaphragm, so much so that the normal convex appearance becomes concave. Inversion of the hemidiaphragm is more commonly seen on the left side. On diascopy of the inverted diaphragm, paradoxical movements are evident, that is, it rises on inspiration and descends on expiration [9].

It is common practice for the chest radiographs to be performed while the patient is standing. However, in critically ill patients, the chest radiographs can be only performed while the patient is lying down, and this could lead to the pleural effusion not being diagnosed. This is due to the fact that pleural fluid is being pulled by gravity and consequently localises in the posterior parts of the thoracic cavity.

Characteristic signs of a pleural effusion on chest radiograph when the patient is lying down are: blunting of the costophrenic angle, increased density of the part of the lung, loss of diaphragmatic silhouette, the presence of apical cap, elevation of a hemidiaphragm, difficulty in spotting vascular structures of the lower lobe, and accentuated small fissure [80]. These signs are not present in patients with a small or moderate pleural effusion.

Serial radiographs of the chest while the patient is lying down after moving the patient from one side to the other side can indicate a presence of mobile fluid and absence of loculations which in malignant pleural effusions enables the determination of a possible pleurodesis site [81].

Using the above mentioned radiological signs, it is possible to differentiate increased density of a pleural effusion from infiltrates for example. First, if the cause of increased density is a pleural effusion vascular structures of the lung will be visible on the radiograph. Any other intra-pulmonary process would cause an obliteration of vascular structures—'the silhouette effect'. Second, if pleural effusion is the cause of increased density, the visible change will be entirely homogenous. Infiltrates caused by an intrapulmonary process are usually less homogenous. Third, air bronchogram is only present when the increased density is a consequence of parenchymal infiltration.

Typical localization of fluid in pleural space of healthy lungs depends on the reverse action of elastic forces. Compared to other parts of the lung, the parts of the lung that are below the effusion exhibit different characteristics of elastic forces. Part of the lung above which the fluid is accumulated has a higher intensity of action reverse elastic forces. Accordingly, atypical localisation of fluid in the pleural space indicates that the part of the pulmonary parenchyma below the effusion is altered. Encapsulated fluid usually produces an image of a bi-convex lens or adopts a lenticular shape. For instance, if lower lobe pathology increases the intensity of action of reverse elastic forces, the fluid will accumulate posteriomedially. Characteristically, the opacity is higher in the axillary line. In some cases, a pleural effusion can mimic middle or lower lobe atelectasis. On the lateral radiographs, the upper border of the density is parallel to the main fissure and is higher in the posterior segments of the thorax, and on moving anteriorly, it descends into the costophrenic sulcus. Lobar or segmental lung collapse can be a cause of pleural effusion development. In these cases, the effusion can mimic pleural adhesion [82].

When the whole hemithorax is opacified, the first priority should be to determine the position of the mediastinum, as the position of the mediastinum depends on the intrapleural pressure. Lower intrapleural pressure will shift the mediastinum to the ipsilateral side of the effusion, (**Figure 2**) while if the pressure is higher on the side of the effusion mediastinum will shift contralaterally, (**Figure 3**) assuming that the mediastinum is not infiltrated by a tumour or an infiltrative process, that is, that it is not fixed. When the mediastinum is fixed, there is no mediastinal

Figure 2. Atelectasis of the left lung with ipsilateral shifting of the mediastinum.

Figure 3. Massive pleural effusion with contralateral shifting of mediastinum.

shift seen. If the mediastinum shifts to the ipsilateral side of the effusion, it can be concluded that the underlying process is an expansive lung disease. Occasionally, in complete atelectasis contralateral lung is a cause of increased retrosternal light, which is clearly seen on a lateral radiograph. This radiological picture corresponds to complete obstruction of the ipsilateral bronchus by a neoplasm. In obstruction of a bronchus, thoracocentesis is not indicated as it is not necessary for diagnostic work up and as it carries a risk of additional increase of the negative intrapleural pressure. Possible complications include pneumothorax and re-expansive pulmonary oedema. In an obstructive lesion, evacuation of a large amount of fluid [more than 1000 ml] is only recommended if a simultaneous measuring if intrapleural pressure is possible [17].

4.5.2. Computed tomography [CT]

CT is efficient in diagnosing pathological alterations of the pulmonary parenchyma and changes that belong to pleural diseases (**Figure 4**). In comparison to standard radiography, pleural alterations are diagnosed much easier on the CT scan and are easily distinguished from the

Figure 4. Lung cancer followed by an ipsilateral effusion.

pulmonary parenchyma and diseases that do not affect the pleura [83]. Collections of fluid or masses have a tendency to adjust to the pleural space. As in the radiograph, the angle between the lesion and thorax helps when trying to differentiate a pleural from a parenchymal change. If the angle between the lesion and thorax wall is sharp, then the change is likely to be a part of the lung parenchyma, while if the angle is blunt, then the lesion is more likely to be of pleural origin. Nevertheless, CT can also be inconclusive. The findings can be similar to the chest radiograph findings, especially when atelectasis or pneumonia are in question, or when a pleural collection forms a sharp angle with thorax wall. Free pleural fluid can form sickle-like opacities in the lowest and posterior parts of the thorax. Fluid collection that is loculated can be seen as a fixed, lenticular opacity. Thickening of the pleura almost invariably points to an exudative effusion [83]. Unreliable signs of pleural invasion are: absence of border area between pleura and primary lesion, blunt angle between the tumour and chest wall, and the presence of a pleural effusion. A probable sign of pleural invasion is if in addition to rib destruction simultaneously the distance between the surface of the tumour and wall of the thorax is less than 3 cm.

The typical features of malignant pleural disease are nodules, irregularity, and pleural thickening >1 cm. It was found that these pleural characteristics discriminated well between malignant and benign disease in a prospective chest radiograph study of 40 patients with suspected malignant effusions, with a sensitivity of 84% and specificity of 100% for malignancy [84]. However, pleural thickening alone was not found to be specific as it was found in malignancy as well as in empyema. Albeit, the presence of pleural nodules was found to be highly specific, it was found

to have only 17% sensitivity—17% of patients with malignant effusion had associated pleural nodules. It is interesting that the half of the patient with malignant effusion had no pleural abnormalities on CT in this study [85]. In patients with lung cancer, even minimal pleural effusions can represent malignant involvement [86]. CT density coefficient is not specific enough to differentiate parenchymal lesion from a solid pleural mass or a serous effusion from blood or pus [87]. In these cases, ultrasound is the investigation of choice and has an edge over CT.

4.5.3. Magnetic resonance imaging [MRI]

Pleural effusion can be identified on an MRI scan; however, MRI is not advised for routine evaluation of malignant effusions.

Collections of pleural fluid are visible as areas of low intensity signal on T1 images, while signal is intensified on T2 images. MRI can sometimes enable the classification of a pleural effusion to transudate, chylothorax, empyema, but in essence, the information gained by MRI is usually insufficient to be of diagnostic use. However, its ability to produce excellent soft-tissue contrast can be useful for detailed evaluation of tumour invasion [88]. MRI is superior to CT in identification of solitary foci on the chest wall, changes of the endothoracic fascia and identifying the invasion of diaphragmatic muscles. MRI can be especially useful in evaluating apices of both hemithoraces.

4.5.4. Positron emission tomography with F-18-fluorodexyglucosae [PET]

PET-FDG imaging accurately detected malignant pleural involvement and the presence of malignant pleural effusion in 16 out of 18 patients and excluded pleural metastatic involvement or malignant effusion in 16 of 17 patients—sensitivity of 88.8%, specificity 94.1%, and accuracy of 91.4%. PET-FDG imaging is a highly reliable and accurate non-invasive test that can differentiate benign from malignant pleural effusions and/or pleural involvement in lung cancer patients and CT findings of suspected malignant pleural effusion [89]. Nonetheless, this form of imaging may not be able to differentiate pleural malignancy from benign inflammation of the pleura, for example, caused by talc pleurodesis [90].

4.5.5. Ultrasound

Thoracic ultrasound can be done with any modern ultrasound machine. Curved ultrasound probe 5–7.5 MHz enables the examination of deep structures of the chest wall. Intercostal spaces are utilised as an acoustic window [91]. When using high frequency probe of 7.5–10 MHz, parietal and visceral pleura are usually seen as echogenic lines, not thicker than 2 mm (**Figure 5**). Diaphragm is visualised as a bright curved line moving upwards or downwards, depending on the respiration phase. High frequency probes improve the resolution in the fields close to source of the echo, enabling differentiation of cystic lesions from solid masses. Ultrasound properties of pleural fluid are best evaluated by changing the form of the fluid during respiration [10]. Ultrasound discovers the presence of fluid in pleural space with an accuracy varying between 87 and 94% [92]. An amount of liquid present in pleural space can be quantified by ultrasonographic examination. Calculated amount of liquid is in better correlation with real amount of fluid present if it is defined by ultrasonographic examination. Amount of liquid

Figure 5. 'Comet tail' sign.

of 1000 ml on radiograms in lateral decubitus correlates to a 30-mm layer of liquid, while the same amount of fluid correlates to ultrasonographic thickness of 40 mm [93].

Ultrasound diagnostics is useful in determining a suitable spot for thoracocentesis, especially in loculated and small effusions [92, 94, 95]. Ultrasound is also helpful in therapeutic thoraco-centesis for measuring effusion the depth of an effusion which makes this procedure safer. At thoracocentesis, the thickness of pleural liquid must not be less than 10 mm. Complications of ultrasound guided thoracocentesis are minimal [94].

Pleural thickenings are presented as constant regions of weak echogenicity. Differentiation of pleural thickening from effusion, tumour, and mesothelioma is uncertain. Respiratory depen-dent configuration change of identified lesion favours a pleural effusion, regular borders favour pleural thickening, and irregular borders favour a diagnosis of a tumour [96]. Plaques produce the image of focal zones of intensive reflection with dense posterior acoustic shadow and usually surrounding non-calcified pleural thickening.

Pleural masses are sonographically presented as masses of unclear limitation, weakly echo-genic, nodal or linearly disseminated along the pleura. Malignant tumour of pleura can infiltrate the chest wall and it leads to poorly visible demarcation of pleural mass from the thoracic wall [97]. Pleural thickening over 1 cm brings forward justified suspicion of a malig-nant tumour. Accompanying pleural effusion is usually visible in a big field which spreads locally and is especially emphasised. The effusion is helpful in tumour identification and it enables differentiation of parietal from visceral pleura. Visceral pleural thickening is rarely

seen. In pleural mass, respiratory motions of lungs during the respiratory cycle are reduced. Very clear echogenic and irregular reflexes are visible on passing towards the ventilated area of lungs.

Metastases might appear as diffuse parietal pleural thickenings. These can be seen as weakly echogenic and moderately echogenic structures; they are oval and easily nodal.

4.6. Cytological and pathohistological examination of pleural fluid and pleural tissue

The percentage of positive cytological results has a wide range, which depends on the type and location of the primary neoplasm, the number of samples being examined, methods and way of sample processing. Results of cytological examination depend on pathohistological type of tumour, number of prepared samples, length of time sample is kept, and interest of cytopathologist.

Malignant cells have several characteristic properties which differentiate them from other cells. They can significantly vary in form and size. They are usually large. The diameter of the nucleus ranges up to 50 μm and their diameter is significantly bigger than, for example, the nucleus of mesothelial cells that are rarely bigger than 20 μm (**Figure 6**). For comparison, small lymphocytes have diameter up to 10 μm. Nucleoli of malignant cells are up to 5 μm in size. Nucleoli of non-malignant cells do not exceed 3 μm. Malignant cells have a high nucleo-cytoplasmic ratio. Morphological analysis itself is not sufficient for differentiation of adenocarcinoma cells from, for example, mesothelioma cells. Mesothelioma cells have a tendency to make papillary groups, and they are multinuclear, with atypia and cell to cell position. Adenocarcinoma cells have a greater tendency to form acini of similar structures. They can form big group of cells. Balloon-like cytoplasmic vacuolization is expressed [98].

Groups of 20 or more benign mesothelial cells can sometimes appear bizarre. Large, vacuolated cells of adenocarcinoma enable differentiation between these two entities. A small number of mitotic figures are often present in benign effusions and the presence of such picture does not point to malignant disease.
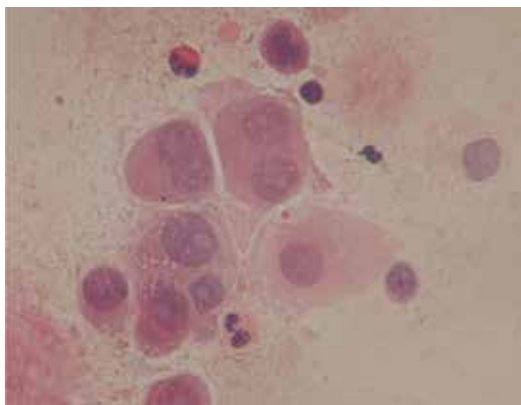

Figure 6. Atypical giant cells in pleural effusion.

Two most important histochemical tests commonly used are alcian blue staining and periodic acid-Schiff. Alcian blue staining enables the detection of acid mucins, which is a specific finding for mesothelioma. Alcian blue staining was positive in 14 out of 19 (73%) patients with mesothelioma, while it was negative in all the patients with adenocarcinoma [99]. Periodic acid-Schiff staining, after diastasis digestion (PAS-D), enables the detection of neutral mucins that have a diagnostic value for adenocarcinoma. PAS-D staining was positive in 27 out of 44 (61%) patients for adenocarcinoma, while it was negative in all patients with mesothelioma [100].

In differentiation of adenocarcinoma from mesothelioma, a whole palette of monoclonal antibodies is used. The same antibodies are found in the presence of benign mesothelial cells, adenocarcinoma, and malignant mesothelioma.

Immunohistochemical staining can be a useful diagnostic tool. Specific markers such as thyroid transcription factor 1 exhibit a high specificity for a primary lung carcinoma, whereas GATA3 has been advocated as a sensitive and specific immunostain for diagnosis breast cancer [101]. Cytological specimens are used for sequencing of mutations of epidermal growth factor receptor (EGFR), [102] and with the use of highly sensitive sequencing such as next-generation sequencing, these markers can be detected even when cytological examination affirms a low percentage of malignant cells or even no malignant cells [103].

Pathologist's challenge can be differentiating mesothelioma from both metastatic adenocarcinoma and non-malignant reactive mesothelium. Using a panel of immunohistochemical stains is now the standard for diagnosing mesothelioma, including using antibodies that stain positively for mesothelioma (WT1, cytokeratin 5/6, calretinin) and those that stain negatively (e.g. adenocarcinoma specific stains such as MOC- 31, CEA, Ber-EP4, and B72.3) [104, 105]. However, it is of note that pleural liquid mesothelin levels can be elevated in a significant number of patients with malignant effusions other than mesothelioma, while mesothelin levels are not elevated in benign effusions. Hence, a high mesothelin level strongly suggests a presence of some form of malignancy [106]. The future of diagnosis might include genetic analysis—either for microarray characteristic of tumours or for characteristics of malignancy (microsatellite, aneuploidy, telomerase DNA methylation, and mutations) [107, 108]. As explained earlier, pleural cells genetic testing may lead to therapeutic choices; for example, establishing an EGFR mutation in malignant pleural cells can the predict response to pertinent EGFR tyrosine kinase inhibitors, like gefitinib or erlotinib [102, 103, 109]. High throughput sequencing technology—a by-product of the Human Genome Project, enables rapid sequencing of either a small percentage of the genome that codes for expressed genes (the exome), or the whole genome. As all cancers are unique, the hope is that this could lead to patient-specific markers and subsequent therapies—for example, designing specific vaccines that 'drive' the immune system of the host to attack that patient's cancer [110].

In previously diagnosed neoplasms, pleural effusion cytology is positive in 50–90% of cases [112]. Falsely positive results range from 0 to 3% [111]. The absence of malignant cells in pleural effusion does not exclude malignancy and this infers the necessity of repeating the cytological investigations.

If repeated cytological analysis is negative, clinical observations and laboratory examinations do not point towards a probable aetiology of pleural effusion, and percutaneous blind biopsy is indicated (**Figure 7**).
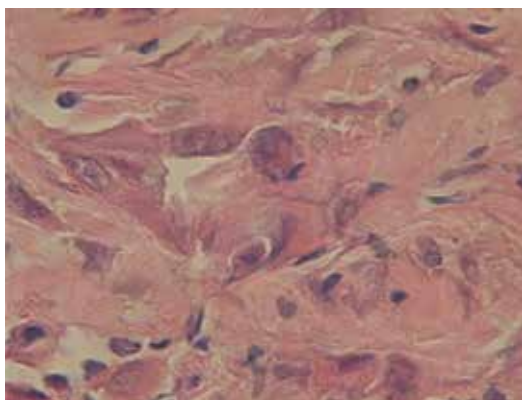
Figure 7. Adenocarcinoma invasivum pleurae HG2, NG3 (EMA × 400).

In seven series of malignant pleural effusions published and analysed in the literature (over 500 recorded cases), cytological analysis of pleural fluid sample had a diagnostic significance ranging from 66 [47] to 76% [57]. Pleural biopsy had positive predictive value of 46% [8]. Combining of these two procedures, cytological and pathohistological analysis, disease was diagnosed in 73% of patients. These data indicate that cytology is the more specific method compared to pleural biopsy, while they also suggest that these tests are complementary and that small samples given by pleural biopsy can be falsely negative.

In a randomised study that compared CT-guided biopsy with closed pleural biopsy using an Abrams needle, CT-guided biopsy was notably more sensitive (87 versus 47%) with a superior negative predictive value (80 versus 44%) [113]. Thoracoscopy is a procedure that is well tolerated and at the same time allows excellent visualisation of the entire pleural surface.

Correct identification of metastatic disease of pleura in nearly 100% of cases is achieved by directed pleural biopsies [117]. This technique provides additional advantages, including the ability to provide the information about the tumour's gross appearance, to provide the information for staging, to drain the pleural space for talc pleurodesis, to lyse adhesions, and the ability to produce large biopsy specimens for genetic and immunohistochemical analysis for molecular markers (e.g. EGFR) if needed. In routine examination, pleural effusion analysis by electronic microscopy has a slight advantage over cytological examination [115].

5. Possible therapeutic modalities of malignant pleural effusions

Treatment of patients with malignant pleural effusion has to be in accordance with the disease prognosis. Asymptomatic patients do not warrant treatment; nevertheless, most patients will go on to develop progressive pleural effusions that will elicit symptoms and require treatment. However, some patients will reach a stable state of pleural fluid formation and removal and these patients not progress to a symptomatic stage.

According to therapeutic possibilities, the doctor's first step is to estimate the patient's performance status. In order to get a clear answer to the question of should the patient with malignant effusion be treated or not treated, the following questions must be answered. Do the current symptoms reduce the patient's quality of life? What was the response of primary tumour to radiotherapy or chemotherapy? What is the performance status? What is the expected survival time? What was the patient's response to initial thoracocentesis? What is the liquid reaccumulation rate after evacuating thoracocentesis? For how long was the patient asymptomatic after thoracocentesis? Did lungs re-expand after drainage? Is the patient in a condition to tolerate intrapleural sclerotherapy?

If one is to follow this line of thought and principle, cooperation with oncologist, cardio-thoracic surgeon, radiotherapist, and everyone involved in the patient's treatment is necessary.

After diagnostic thoracocentesis is performed, therapeutic thoracocentesis follows (without intrapleural medication) which probably will not result in long-term control of pleural effusion [79, 116]. Possible complications of repeated thoracocentesis are secondary infections, loss of proteins, artificial pneumothorax, effusion loculation, and trapped lung.

5.1. Significance of specific oncological treatment

One of the important principles of oncological treatment is primary implementation of radio and/or chemotherapy. In cancers which have a high probability of being highly sensitive to systemic chemotherapy, for example, lymphoma, chemotherapy is the treatment of choice [79]. Of course, thoracocentesis is necessary in the initial phase of diagnostics and treatment, for both cytological examination and alleviating symptoms of respiratory distress.

Patients with an effusion and a tumour that is refractory to chemotherapy should have thoracontesis performed every 3-4 days for symptomatic relief. In NSCLS, colon and pancreatic cancer, favourable effects of primary systemic chemotherapy and/or radiotherapy are not expected. Intrapleural therapy will probably be indicated for these patients.

5.2. Treatment modalities—therapeutic approach

Moribund patients and patients in the preterminal phase of disease, with the symptoms of respiratory distress thoracocentesis is an urgent therapeutic procedure for alleviating current symptomatology. Aggressive therapy is not recommended in such patients. In view of possible complications during repeated thoracocentesis, a certain number of doctors reserve thoracocentesis only for moribund patients as a type of a short-term symptomatic therapy.

Patients who have a good performance status and longer survival time is expected, chemotherapy and/or radiotherapy is administrated after thoracocentesis. Generally, systemic chemotherapy produces disappointing results when it comes to control of malignant pleural effusion. Since the adverse effects from radiation pneumonitis outweigh the possible benefits of therapy, hemithorax radiation is contraindicated in malignant pleural effusion from lung cancer as a rule (**Figure 8**).
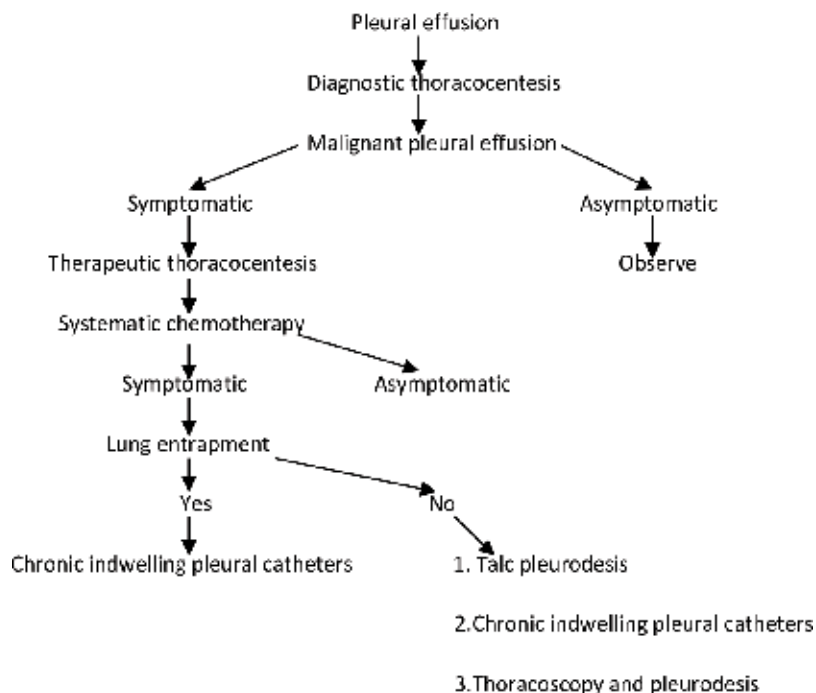
Figure 8. Diagnostic and therapeutic algorithm in malignant pleural effusions.

Drainage or intrapleural therapy is indicated if previous therapeutic protocols produce no results, that is, if they did not enable the control of pleural fluid production. Reaccumulation of larger amount of liquid leads to respiratory distress and/or worsening of clinical picture.

Pleurectomy, shunts, and other aggressive surgical interventions are reserved for patients refractive to intrapleural therapy. Pleurectomy and/or pleural abrasion are highly effective in obliteration of pleural space and in malignant pleural effusion control [117]. Therefore, this procedure is reserved for patients who have a reasonably long expected survival time and are in good general condition or who have failed a sclerosing agent therapeutic procedure. Pleuroperitoneal shunt has been demonstrated to be both safe and effective [118]. The shunt can be especially beneficial in refractory chylothorax where it allows recirculation of chyle [119]. Hyperthermic intrathoracic chemotherapy perfusion (HITHOC) combined with cytoreductive surgery can be performed in selected patients with acceptable mortality and morbidity rates [120].

5.3. Use of thoracic drain

Primary therapeutic task for treatment of patients with malignant effusion is alleviating dyspnoea. Reaccumulation of fluid sometimes can be controlled by intrapleural instillation of medication. In controlling the usual techniques of thoracocentesis, needle aspiration, and drainage are rarely efficient [79]. Treatment is basically palliative, disregarding the stage of disease and condition of the patient. Drainage postpones respiratory distress, but it does not prevent reaccumulation of liquid, dyspnoea, and pleural pain. Therefore, all patients with

notable symptomatology, except moribund and those patients in preterminal stage of disease, are recommended to have drainage of pleural space with instillation of therapeutic solution. Intrapleural therapy often produces satisfactory outcomes with in regard of long term palliation of respiratory symptoms by reducing or eliminating pleural liquid formation.

In loculated effusions, drainage is not successful. This is due to the fact that the lung never re-expands sufficiently so that visceral and parietal pleura are in contact. In this case, intrapleural therapy is not recommended.

Instillation of thoracic drain is a safe and successful surgical procedure enabling at the same time diagnostics and treatment of malignant pleural effusion. If it is performed correctly, the method is relatively painless.

After placing the drain, it is necessary to perform control radiographs in order to check the correct position of the drain, exclude pneumothorax, establish lung expansion, and amount of liquid that is possibly retained.

Lung expansion is necessary to achieve in order to close the space previously filled with liquid and to bring pleural surfaces into contact.

Failure of pleurodesis is associated with abnormal lung expansion that is detected with pleural manometry. A pleural space elastance greater than 19.0 cm H_2O/L during the evacuation of first 500 ml of pleural liquid was found to predict 100% pleurodesis failure at 1 month [121]. Lung expansion abnormalities detected during later stages are indicative of immediate or delayed pleurodesis outcomes.

Prerequisite for pleurodesis is that the effusion is not loculated, that patient has a good performance status and that expected survival time is longer than 4 weeks. After adequate evacuation of liquid, medicines are instilled into pleural space by bolus. Whether the procedure is done with or without active suction, fast decompression should be avoided. Consequences of fast decompression are severe pain, pulmonary shock, mediastinal shift, and pulmonary oedema of the re-expanded lung. If the effusion is massive, 1000–1500 ml can be evacuated. Drained should be intermittently clammed, especially in the early phase of suction. This way, hemodynamic stabilisation of patient is ensured. Drainage is continuous while daily amount of aspirated content is higher than 100 ml. By irritating pleura and lungs, every drain will produce around 50 ml of pleural liquid during 24 h. Drainage gives satisfactory results in less than 20% of patients [69, 116]. Nevertheless, pleurodesis in an outpatient setting using small-bore catheters can be successfully performed with decreased cost and morbidity.

The utilisation of indwelling catheters [Denver Biomaterials, Golden, PleurX, Colorado] has attained popularity due to it being an outpatient procedure, which allows the patient and family to manage the pleural effusion in a timely fashion at home. For symptomatic refractory or recurrent malignant pleural effusion, these catheters have grown to be the mainstay of treatment in most centres in the United States. This is due to their ability to successfully palliate the symptoms of dyspnoea regardless of the presence of lung entrapment. Spontaneous pleurodesis is developed by approximately 50% of patients by 2 months [122, 123].

5.4. Medicine choice for intrapleural therapy

It is presumed that medications such as tetracyclines, talc, and nitrogen mustard, etc. cause inflammation of pleural surfaces. Inflammation leads to obliteration of pleural space disabling reacummulation of liquid [pleurodesis]. The mechanism of action of 5-fluorouracil effect is even less clear. Agents like cisplatin and cytosine arabinoside locally achieve high concentrations; thus, they are assumed to have a direct cytoreductive effect [116].

Choice of medication for pleurodesis partly depends on clinical, and partly on non-clinical parameters.

5.4.1. Nitrogen mustard

Nitrogen mustard (mechlorethamine) has been used in intrapleural therapy since 1949 and it is one of the first medications used in control of malignant pleural effusions [124]. Medicine efficiency is different and is accompanied by a large number of side effects such as chest pain, nausea, and vomiting [124, 125].

5.4.2. Talc

Talc is one of the oldest and most efficient medicines [79, 125]. Talc is instilled into the pleural space as a suspension or powder (**Figure 9**). Insufflation of talc powder has proven to be more efficient than instillation of the suspension [125]. Following talc poudrage and slurry, fever is a common occurrence, occurring 16–69% of the time. Complications that have also been reported with talc usage include arrhythmia, empyema, respiratory failure including pneumonitis, and adult respiratory distress syndrome (ARDS).

5.4.3. Quinacrine

Quinacrine is an antimalarial medication which has been recommended in therapy of malignant effusions for a period of time. Its use is completely abandoned now [79].

5.4.4. Biological agent—corynebacterium parvum

Biological agent—corynebacterium parvum in dose from 5 to 10 mg was promising at first; however, randomised study which compared its efficiency with efficiency of tetracycline did not show a statistically significant efficiency [126, 127].

5.4.5. Tetracycline

Tetracycline is a very popular medicine in intrapleural therapy because of its efficiency, affordability, and ease of use. Recommended dose for instillation is between 500 mg and 3 g of diluted tetracycline in 50–100 ml of normal saline [114, 127]. The most common side effects, in about 30–40% patients, are fever and moderate to severe chest pain which requires premedication [79]. Tetracycline is efficient in prevention of pleural effusion recurrence as well. Crucial factor for treatment success is fast and complete dispersion of tetracycline in pleural space [125].
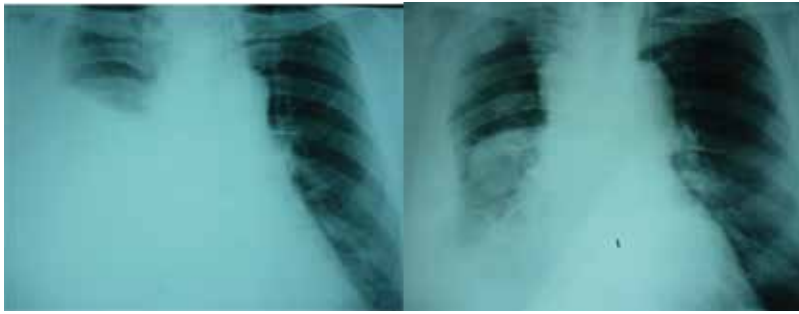
Figure 9. CXR before and after pleurodesis.

5.4.6. Bleomycin

Bleomycin is an antitumor, antibiotic producing significant results in prevention of pleural liquid reaccumulation [128]. Usual dose is 60 U in 100 ml D5W or normal saline [129, 130]. Systemic reabsorption is limited. About 40–45% of medicine is resorbed via pleura. This dose of medicine does not supress the bone marrow; thus, bleomycin can be used simultaneously with chemotherapy and radiotherapy and it can be used in patients that suffer from myelo-suppresion [129].

In prevention of malignant pleural effusion, recurrence bleomycin is more efficient than tetracycline. Average time of effusion recurrence after tetracycline therapy (dose of 1 gram) was 32 days and 46 days for bleomycin (dose of 60 U) [129]. Series of three to four daily instillations of bleomycin is used in refractory effusions with a reduction in dosing from 60 to 30 U [131] is recommended.

5.4.7. Other medicines

Other medicines used for pleurodesis are thiotepa and 5-fluorouracyl, but they are less efficient than above mentioned medications. It is assumed that NaOH implementation achieves chemical pleuritis [132]. Doxorubicin has a similar effect to nitrogen mustard [121].

After pleurodesis, reaccumulation of liquid occurs in around 20–30% patients. If this is a small amount of fluid and is well tolerated by the patient, further treatment is usually not necessary.

The most efficacious methods in control of the effusion are pleural abrasion, chest tube drainage with sclerosing agent instillation, and pleurectomy. Mixed intravenous and intrapleural chemotherapy, with air therapy has produced response in 55% of patients, complete remission has resulted in 7%, partial remission in 48%, in 22% disease stabilisation has been achieved, and disease progression has been recorded in 22% of patients [133].

5.5. Therapy associated pain

After intrapleural administration of sclerotherapy, pain might occur. After drainage of 15–20 ml, 2% lidocaine is instilled via drain or catheter. The patient needs to breathe deeply and cough mildly for better distribution of anaesthetic over the surface of pleural space. Five to ten minutes before tetracycline is intravenously injected, 75–150 μg fentanyl is administrated, and

5–10 mg of morphine is given after that. Fentanyl acts very quickly, as morphine takes over the extended analgesic effect. Fentanyl is slowly injected, over 5–7 minutes. If an unwanted reaction to fentanyl is expected, naloxone is given. Doctor must make sure that drain or flexible catheter is vertical and that intrapleural agent from the drain interflows into pleural space. In the analgesic therapy, results are achieved with less strong medications; therefore, premedication with morphine 10 mg or meperidine 75–100 mg intramuscularly or subcutaneously is recommended. Just before tetracycline solution instillation, 20 ml of 2% lidocaine is given intrapleurally. Morphine, fentanyl as well as other analgesics can have a depressive effect on respiratory centre and therefore, these principles of pain therapy cannot be applied in elderly patients, patients who are predisposed to respiratory depression and those with a bad general condition.

6. Conclusion

PMPE were equally present in all pathohistological types of lung cancer, while MPE were most common in lung adenocarcinoma. The diagnostic yield of pleural fluid cytology and closed pleural biopsy combined was more than 90%. Most commonly used therapeutic procedures were thoracocentesis and pleurodesis. PMPE were not a contraindication for explorative thoracotomy.

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Diagnosis of Lung Cancer: What Metabolomics Can Contribute

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Abstract

The reprogrammed metabolism of cancer cells reflects itself in an alteration of metabolite concentrations, which in turn can be used to define a specific metabolic phenotype or fingerprint for cancer. In this contribution, a metabolism-based discrimination between lung cancer patients and healthy controls, derived from an analysis of human blood plasma by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, is described. This technique is becoming widely used in the field of metabolomics because of its ability to provide a highly informative spectrum, representing the relative metabolite concentrations. Cancer types are characterized by decreased or increased levels of specific plasma metabolites, such as glucose or lactate, compared to controls. Data analysis by multivariate statistics provides a classification model with high levels of sensitivity and specificity. Nuclear magnetic resonance (NMR) metabolomics might not only contribute to the diagnosis of lung cancer but also shows potential for treatment follow-up as well as for paving the way to a better understanding of disease-related diverting biochemical pathways.

Keywords: metabolomics, human blood plasma, metabolic phenotype, ^{1}H-NMR spectroscopy, metabolite spiking, multivariate OPLS-DA statistics, lung cancer, cancer cell metabolism, biomarker

1. Introduction

Metabolomics, or metabolite profiling, comprises the study of the entire spectrum of low-molecular weight metabolites and their cellular processes in a biological system [1–4]. Next to

a large number of studies exploring the use of metabolomics in the field of disease diagnosis and prognosis, its application is also extended to other research areas such as toxicology [5], nutrition [6], microbiology [7], and drug discovery [8]. Together with high prevalence diseases such as diabetes [9], obesity [10–12], and neurological and cardiovascular disorders [13, 14], different types of malignant diseases including breast [15, 16], colorectal [17, 18], and lung cancer [19–24] are being extensively examined by using a metabolomics approach.

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), which can possibly be connected to a gas- or liquid chromatography system (GC-MS/LC-MS), are the analytical techniques that are primarily used in the field of metabolomics [25–27]. While ^{13}C nuclei can be very useful in contribution to metabolite identification by NMR, the proton (^1H) nucleus is mostly studied in metabolomics NMR experiments [28]. The ^1H nucleus is omni-present in metabolites, shows the highest relative sensitivity, and has a natural abundancy of 99.98%. ^1H-NMR spectroscopy is a noninvasive technique that needs no sample extractions and that enables the identification and quantification of metabolites in biofluids as well as in tissues and therefore is becoming widely used in the field of metabolomics [29]. Despite that ^1H-NMR is less sensitive compared to MS, it has many advantages: nondestructive, easy quantification, low cost per sample, minimal sample preparation requirements resulting sub-sequently in an excellent reproducibility and rapid high-throughput data acquirement [30]. In a single run of a few minutes, the ^1H-spectrum from one sample provides information regarding the relative concentrations of all present metabolites. The metabolic phenotype provides a representative snapshot of an individual's metabolic state and therefore enables the determination of cellular processes altered by disease [2].

Metabolites from a number of different diagnostic biofluids are already examined in multiple studies, mostly involving human blood plasma, serum, or urine [1, 22, 31, 32]. In parallel with bio-fluids, intact tumor tissues are frequently evaluated since intra-tumor heterogeneity is currently one of the major causes of treatment failure [33, 34]. To that end, high-resolution magic angle spinning NMR (HR-MAS NMR) as an analytical approach is gaining great attention [35–38].

This review intends to point out the results of ^1H-NMR metabolic profiling of lung cancer patients acquired by our research group and further explores the benefits which this method might deliver to contribute to an optimal treatment for lung cancer patients.

2. Methods

2.1. Sample collection and preparation

Experimental design focused on the analysis of fasting venous blood samples from lung cancer patients. Importantly, exclusion criteria were (i) not fasted for at least 6 h; (ii) fasting blood glucose concentration ≥ 200 mg/dl; (iii) medication intake in the morning of blood sampling, and (iv) treatment or history of cancer in the past 5 years, as described in the study of Louis et al. [20]. The blood samples were collected in lithium-heparin tubes and stored at 4°C within 5 min. Plasma aliquots were obtained after centrifugation at 1600 g for 15 min within 8 h after collection. Plasma sample preparation included a centrifugation step at 13,000 g for 4 min at 4°C and dilution of 200 μl of the supernatant with 600 μl deuterium oxide (D_2O) containing

0.3 µg/µl trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) as a chemical shift reference of the spectra. After presaturation for water suppression, the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to acquire slightly T2-weighted spectra on a 400 MHz (9.4 Tesla) NMR spectrometer [1].

2.2. Spectral processing

2.2.1. Binning

Before applying multivariate statistics, the data acquired by ^{1}H-NMR analysis should be pre-processed. Preprocessing of data usually includes phasing, baseline correction, alignment, and normalization. In addition, the spectrum has to be divided into regions of which the integration value (i.e., area under the peak) can be used as a variable for the statistical analysis. Binning or bucketing is a commonly used technique to produce such a reduced set of variables by segregating the spectrum [39]. In point-wise binning, the spectrum is divided into so-called equally-sized bins. An important limitation of this method is the possible splitting of peaks, resulting in a loss of differentiating power and possibly data misinterpretation. To overcome this, another methodology based on spiking of the plasma with known metabolites is proposed. This approach describes how the ^{1}H-NMR spectrum is divided into well-defined variable-sized integrations regions, being the variables for multivariate statistical analysis [40].

2.2.2. Spiking methodology

To obtain a correct signal assignment, ^{1}H-NMR spectra of reference plasma samples to which a known metabolite was spiked, were acquired. Hereto, stock solutions were prepared by dissolving a relevant concentration of a known metabolite in a reference plasma sample. Reference plasma can be obtained by pooling the plasma of several blood samples from a healthy person. Next, a small amount of stock solution can be added to a reference plasma NMR sample (e.g., 10 µl stock solution to 200 µl reference plasma and 600 µl D_2O containing the TSP reference). This procedure can be repeated for all metabolites of interest, using a fresh reference sample for each metabolite. The outcome of these spiking experiments allows an accurate identification of the chemical shifts and J-coupling patterns. On our 400 MHz (9.4 Tesla) NMR spectrometer, the described spiking method led to a segmentation of the spectra in 110 well-defined integration regions [40]. After integration and normalization (relative to the total integrated area, with exclusion of the contributions of TSP and water), these integration regions could be used as variables for multivariate statistical analyses.

2.3. Multivariate statistics

The statistics were carried out by using supervised orthogonal partial least squares discriminant analysis (OPLS-DA) to train and validate a classification model which enables optimal discrimination between lung cancer patients and a control population. The statistical classifier was constructed after detection and removal of outliers in the training data set via unsupervised principle component analysis (PCA). In addition, PCA was also conducted to visualize significant intrinsic clusters in the case–control data set upon which identification of possible confounders was based.

Model characteristics such as the total explained intra- (R²X(Cum)) and intergroup (R²Y(Cum)) variation were examined together with sensitivity and specificity values in order to evaluate strength performance of the OPLS-DA classifier. Predictive ability (Q²(Cum)) of the model was demonstrated by cross-validation of the training set as well as by application of the model to an independent validation cohort.

3. Results

3.1. Detection of lung cancer

The assigned and normalized integration regions of the ^{1}H-NMR spectrum reflect the relative metabolite concentrations and thus represent the metabolic phenotype. Therefore, they can be used as variables for multivariate OPLS-DA statistics in order to discriminate between lung cancer patients and healthy controls. By applying this methodology on lung cancer plasma samples, a classification model that enables discrimination between those two groups was trained. Hereto, a large training cohort consisting out of 233 lung cancer patients and 226 controls was used. Characteristics of the subjects included in the training and validation cohort are summarized in **Table 1**. The trained OPLS-DA classifier resulted in a correct classification of 78% of the lung cancer patients and 92% of the control group (**Figure 1A**) [19]. To affirm that the discrimination was purely due to differences in plasma metabolite concentrations, PCA was conducted to exclude possible confounders. By means of PCA score plots, it was confirmed that gender, smoking status, disease, and chronic obstructive pulmonary disease (COPD) are no confounders [19].

While these results definitely support the applicability of this methodology for the detection of lung cancer, no clear differentiation between tumor stages or histological subtypes could be detected yet, that is, none of the trained OPLS-DA models already showed significant clustering of different tumor stages or histological subtypes. This probably is due to the limited number of lung cancer patients in the subgroups and the diffuse character of the subgroups formed on the basis of histology and clinical tumor stage. However, the ability of a constructed OPLS-DA model to discriminate between 76 stage I lung cancer patients and 76 randomly selected controls with 74% sensitivity and 78% specificity strongly suggests that plasma metabolite phenotyping reveals the presence of lung cancer already during early stadia of tumor development (**Figure 2**) [19].

3.2. Validation of the classification model

Importantly, after training of a promising classification model, confirmation of the validity of the model needs to be considered. When the metabolic fingerprint of a large cohort of patients and controls is available, this can be realized by applying the model on an independent validation cohort consisting out of an independent group of both lung cancer patients and controls. In this study, an independent cohort of 98 patients with lung cancer and 89 controls was used for validation of the trained model classifier. The trained model shows a high predictive accuracy with a sensitivity of 71% and a specificity of 81% (**Figure 1B** and **C**) [19].

	Training cohort		Validation cohort	
	C	LC	C	LC
Number of subjects, N	226	233	89	98
Gender, N (%)				
Male	119 (53)	160 (69)	44 (49)	66 (67)
Female	107 (47)	73 (31)	45 (51)	32 (33)
Age, yrs.	67 ± 11	68 ± 10	69 ± 10	64 ± 9
(range)	(38–88)	(36–88)	(47–89)	(45–83)
BMI, kg/m²	28.3 ± 5.0	25.8 ± 4.5	28.4 ± 5.7	26.2 ± 4.7
(range)	(18.7–46.7)	(17.5–41.8)	(16.2–52.0)	(16.8–38.5)
COPD, N (%)	39 (17)	119 (51)	9 (10)	35 (36)
Taking lipid-lowering medication, N (%)	124 (55)	122 (52)	56 (63)	39 (40)
Diabetes, N (%)	23 (10)	40 (17)	20 (22)	12 (12)
Smoking habits				
Smoker, N (%)	47 (21)	113 (49)	15 (17)	48 (49)
Ex-smoker, N (%)	102 (45)	110 (47)	36 (40)	46 (47)
Non-smoker, N (%)	77 (34)	10 (4)	38 (43)	4 (4)
Pack years	16 ± 24	33 ± 21	13 ± 18	38 ± 21
(range)	(0–175)	(0–125)	(0–60)	(0–150)
Laterality				
Left, N (%)		103 (44)		40 (41)
Right, N (%)		119 (51)		54 (55)
Bilateral, N (%)		6 (3)		4 (4)
Unknown, N (%)		5 (2)		0 (0)
Amount of tumors, N		239		102
Histological subtype				
NSCLC-Adenocarcinoma, N (%)		91 (38)		46 (45)
NSCLC-Squamous carcinoma, N (%)		66 (28)		29 (28)
NSCLC-Adenosquamous carcinoma, N (%)		5 (2)		1 (1)
NSCLC-Carcinoid, N (%)		5 (2)		0 (0)
NSCLC-NOS, N (%)		8 (3)		6 (6)
SCLC, N (%)		30 (13)		15 (15)
Unknown, N (%)		34 (14)		5 (5)
Clinical stage according to 7th TNM edition				
IA, N (%)		55 (23)		12 (12)
IB, N (%)		21 (9)		5 (5)

	Training cohort		Validation cohort	
	C	**LC**	**C**	**LC**
IIA, *N* (%)		11 (5)		7 (7)
IIB, *N* (%)		15 (6)		4 (4)
IIIA, *N* (%)		48 (20)		17 (16)
IIIB, *N* (%)		26 (11)		12 (12)
IV, *N* (%)		63 (26)		45 (44)

BMI: Body mass index; C: controls; COPD: chronic obstructive pulmonary disease; LC: lung cancer patients; NOS: not otherwise specified; NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer; and TNM: tumor, node, metastasis.

Table 1. Summary of the characteristics of the subjects included in the training and validation cohort.
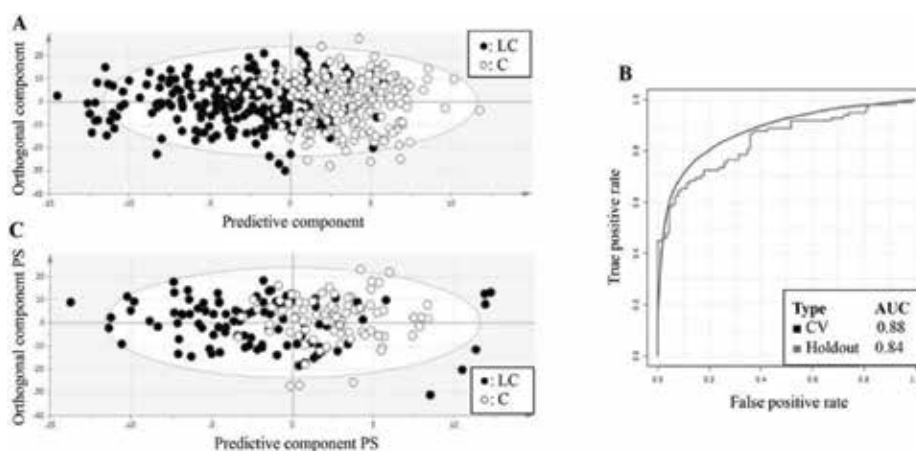
Figure 1. OPLS-DA score plots, resulting from the classification of the training cohort of 233 lung cancer patients and 226 controls (A) and the independent validation cohort of 98 lung cancer patients and 89 controls (C). The AUC of ROC curves confirms the predictive ability of the classification model by cross-validation of the training cohort and an independent validation model (B). AUC: Area under the curve; C: controls; CV: cross-validation; LC: lung cancer patients; PS: predicted scores; and ROC: receiver operating characteristic.
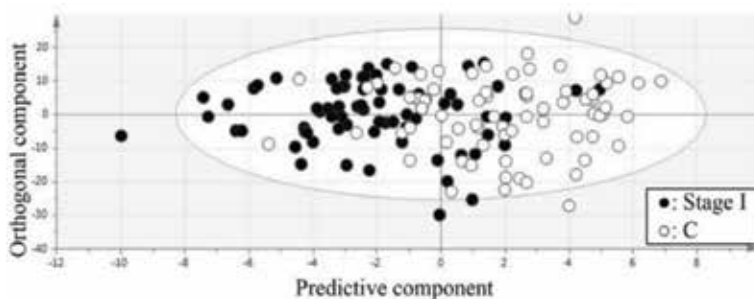
Figure 2. OPLS-DA score plot, resulting from the classification of 76 stage-I lung cancer patients and 76 randomly selected controls of the training cohort. C: Controls.
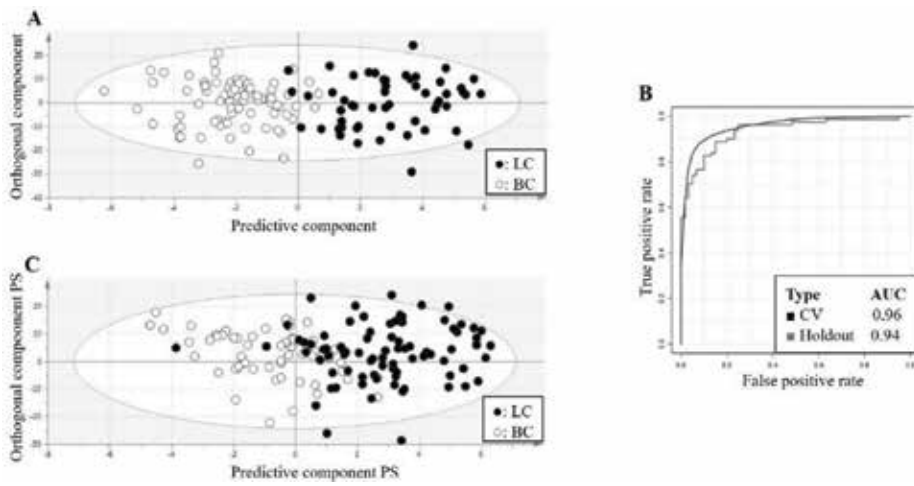
Figure 3. OPLS-DA score plots, resulting from the classification of the training cohort of 54 lung cancer patients and 80 breast cancer patients (A) and the independent validation cohort of 81 lung cancer patients and 60 breast cancer patients (C). The AUC of ROC curves confirms the predictive ability of the classification model by cross-validation of the training cohort and an independent validation model (B). AUC: Area under the curve; BC: breast cancer patients; LC: Lung cancer patients; PS: predicted scores; and ROC: receiver operating characteristic.
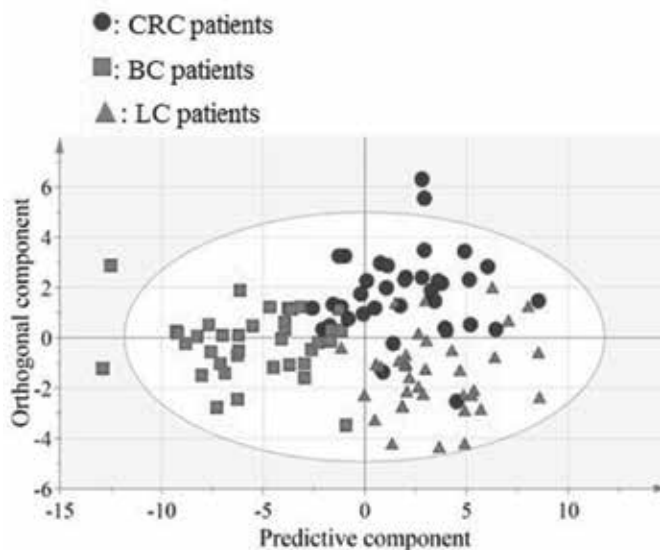
Figure 4. OPLS-DA score plot, resulting from the classification of a population of lung-, breast- and colorectal cancer patients, each group consisting of 37 individuals. CRC: Colorectal cancer patients; BC: breast cancer patients; and LC: lung cancer patients.

3.3. Differentiation between cancer types

To further illustrate the potential of the methodology described above, the following paragraph demonstrates that different cancer types are characterized by a specific metabolite profile. Hereto, the same workflow was applied on a data set of 54 lung cancer patients and

80 breast cancer patients. Again, the segmentation of the spectrum was based on metabolite spiking and OPLS-DA statistics were used to train a classification model, this time in discriminating lung cancer from breast cancer. The resulting model allows a correct classification of both cancer types with a sensitivity of 93% (93% of the 54 lung cancer patients were correctly classified) and a specificity of 99% (99% of the 80 breast cancer patients were correctly classified) (**Figure 3A**). Validation of the model by applying it on an independent cohort of 81 lung cancer patients and 60 breast cancer patients confirmed these findings and shows a sensitivity of 89% and a specificity of 82% (**Figure 3B** and **C**) [20]. Another recent study explored these promising results by establishing an OPLS-DA classification model that allows discrimination between three different types of cancers, that is, lung, breast, and colorectal cancers. After ¹H-NMR measurements of 37 plasma samples of each patient group, multivariate statistics revealed that each type of cancer was represented by a specific metabolic signature (**Figure 4**) [41]. Since the metabolic phenotype allows a clear differentiation between different cancer types, it can be assumed that the metabolic profile should not be considered as a general cancer marker but rather as a distinguishing characteristic of a specific cancer type.

4. Reorganization of metabolic pathways

The metabolites that contributed the most to the differentiation between lung cancer patients and healthy controls were identified and selected based on their variable importance for projection (VIP) value by means of an S-plot. The variables on the wings of the S-plot are the ones with the strongest contribution to the model and the highest statistical reliability [42]. Metabolic phenotyping of blood plasma shows that lung cancer patients are characterized by elevated glucose and decreased lactate levels, which implies an increased gluconeogenesis. This enhanced gluconeogenesis reflects the reaction of the human body to the Warburg effect or aerobic glycolysis in which, even in normoxic conditions, cancer cells rely on fermentation, that is, glycolysis leading to lactate production via fermentation of pyruvate. The Warburg effect, which takes place in cancer cells, can be observed in tumor tissue by means of ¹H-NMR as shown by Rocha et al. They demonstrated that lung tumors of different histological subtypes are all characterized by lowered glucose whereas lactate levels are increased, which is supported by the significantly enhanced glycolytic activity of cancer cells compared to normal cells [23]. Moreover, lung cancer patients show decreased phospholipid plasma levels, pointing to an increased lipogenesis and enhanced membrane synthesis, which is correlated with increased proliferation of cancer cells [43–46]. Other metabolites with an increased concentration in lung cancer patients compared to controls are N-acetylated glycoproteins, β-hydroxybutyrate, leucine, lysine, tyrosine, threonine, glutamine, valine, and aspartate. Contrarily, metabolites showing a decreased concentration in lung cancer patients are alanine, sphingomyelin, citrate, chlorinated phospholipids (e.g., phosphatidylcholine), and other phospholipids [19].

5. Metabolomics in daily clinical practice

5.1. Effect of the NMR magnetic field strength

Evaluation of the advantages versus limitations of NMR spectrometers with higher magnetic field strength was accomplished by comparing the results obtained for the same plasma samples

on both a medium-field (9.4 Tesla; 400 MHz) and high-field (21.1 Tesla; 900 MHz) NMR spectrometer. For a 900 MHz spectrum, an improved resolution as well as a higher signal to noise (S/N) ratio is observed as compared to a 400 MHz spectrum (**Figure 5**) [47]. Because of these improved characteristics, measurements with a high-field spectrometer enable to define the integration regions more accurately using spiking experiments, resulting in less signal overlap and therefore in a larger number of integration regions that are representative for a single metabolite. Yet, discriminative power of both high- and medium-field spectra is rather comparable. These findings are in line with the study of Bertram et al., who demonstrated that the prediction performance and thus obtained information out of the spectra meant for diagnosis strongly increases when shifting the magnetic field strength from 250 to 500 MHz, whereas the effect of further increasing the magnetic field strength from 500 to 800 MHz appeared less strong when group discrimination is concerned [48]. However, analysis with a high-field spectrometer can be the preferred choice for the detection and identification of new, low-concentration metabolites and therefore can contribute to a better understanding of the underlying disturbed biochemical pathways of disease [47]. A drawback is the high cost of high-field spectrometers, which raises strongly with the magnetic field strength. By comparison, the cost of a 400 MHz spectrometer is in the order of €300,000 while a 900 MHz spectrometer can reach the cost of €2,750,000. The need of a supplementary cryoprobe can raise these estimated amounts even more with €200,000 [47]. In addition, such instruments demand for an isolated building for its housing, which is less practical in a clinical setting. Taken all into account, medium-field (400–600 MHz) spectrometers will probably become the preferred instruments for future application in clinical metabolomics.

5.2. Precision medicine

The contribution of metabolic phenotyping toward the clinical environment, often referred to as pharmacometabolomics, can encompass the entire patient journey, starting from an improved screening selection and earlier diagnosis to a follow-up for treatment response prediction and enhanced personalized choice of therapy [49]. Despite several challenges that accompany the implementation of such a unique innovative technique, for example, biomarker validation and cost-effectiveness [49, 50], the authors are highly convinced that metabolism-based biomarkers carry the potential to significantly contribute to future daily standard clinical practice.
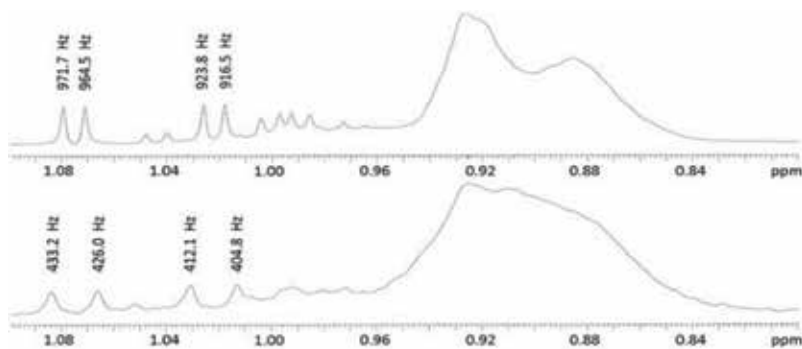

Figure 5. Comparison of the ¹H-NMR spectra of human blood plasma acquired at a high-field (900 MHz) (top) and medium-field (400 MHz) (bottom) spectrometer. Both spectra are zoomed-in between 0.80 and 1.10 ppm. The top spectrum shows an increased resolution and improved S/N ratio. The paired labeled peaks each represent a methyl group of the amino acid valine. ppm: Parts per million.

For lung cancer, metabolic phenotyping by means of ^{1}H-NMR can further be useful in preceding low-dose computed tomography (LDCT) scanning as a tool to deliver additional and complementary risk factors for a better selection of high-risk individuals. Currently, selection of those individuals is primarily based on age and smoking status/history [51]. As an outcome of the National Lung Screening Trial, it is stated that mortality is significantly reduced when screening with LDCT occurs [52]. Although sensitivity levels of LDCT screening are high and the number of diagnoses in early stadia increases, the positive predictive value of LDCT is currently still low [53]. Other drawbacks of LDCT screening are the high rate of false positive results, the high risk of overdiagnosis and consequently additional radiation exposure due to avoidable diagnostic tests [54]. In order to meet with the raising interest in improving the accuracy of risk prediction, promising clinically relevant diagnostic biomarkers which can add predictive value to existing models are indispensable [55, 56]. Therefore, a noninvasive blood-based screening test in complement with LDCT would be a valuable tool to reduce the number of individuals undergoing unnecessary and sometimes harmful follow-up treatments. Likewise, in a next phase, identification of prognostic biomarkers could assist in the tracing of early-stage lung cancer patients who would most likely benefit from current therapies, for example, surgery with curative intent or adjuvant chemotherapy [57].

Next to the discovery of diagnostic and prognostic biomarkers, metabolic profiling is being extensively examined for its use in prediction of individual therapy response [58–61]. Personalized treatment will contribute to a reduction of adverse reactions by (i) prediction of the patient's response and (ii) administration of the most efficient drug dose. Moreover, longitudinal monitoring of patients allows to track post-interventional outcome or deviations in response and therefore can assist in paving the way toward long-term personalized health [49].

6. Conclusion

Analysis of metabolic changes in blood plasma by ^{1}H-NMR spectroscopy allows to significantly discriminate between lung cancer patients and healthy controls. Additionally, metabolic phenotyping supports detection of lung cancer in all stages and enables differentiation between different cancer types such as breast and lung cancers. This indicates that a metabolomics approach can actively contribute to lung cancer diagnosis, even in early stages of tumor development. For daily clinical practice, where the main goal is to correctly classify patients, a medium-field (400–600 MHz) NMR spectrometer can provide sufficient discriminative power to perform clinical metabolomics. For research purposes, on the other hand, where disease-related disturbed pathways deserve a more extensive analysis, high-field NMR (e.g., 900 MHz) spectra are preferred. The ability of high-field NMR to observe a larger number of metabolites that are represented by a nonoverlapping signal, permits a deeper look into the underlying affected metabolic pathways. We show that increased glucose levels are observed while lactate levels are decreased in blood plasma of lung cancer patients. These aberrant metabolite concentrations indicate an increased gluconeogenesis as counteraction of the body to the Warburg effect in the cancer cells. Moreover, the fact that cancer cells manage an enhanced membrane synthesis can be confirmed by the lowered plasma levels of phospholipids.

Encouraged by all these promising results, the authors strongly believe that ^{1}H-NMR-based metabolic fingerprinting will become widely clinically implemented by serving as (i) an

additional screening tool for lung cancer, (ii) a procedure to define complementary risk factors for current risk models toward an improved selection of lung cancer patients eligible for LDCT, and (iii) an innovative method to better characterize lung cancer patients in order to provide them with the best treatment strategies available.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Insights on Treatment of Lung Cancer

Immunotherapy in Advanced Lung Cancer Treatment

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Additional information is available at the end of the chapter

Abstract

Despite the improvement in overall survival (OS) by platinum-based chemotherapy (NSCLC Meta-Analyses Collaborative Group, 2008), prognosis remains unsatisfactory for patients with advanced non-small cell lung cancer (NSCLC). We discuss in this chapter the new era of advanced lung cancer systemic therapy represented by immunotherapy. First of all I presented one of the modalities of immunological diagnostics based on new technology. The mechanism of action of the immunoagents is shortly described. In the in most part of the chapter, the main immunotherapeutic agents used in lung cancer immunotherapy are analyzed: vaccines, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) inhibitors, and checkpoint inhibitors. In the end of the chapter, the combination between immunotherapeutic agents is discussed.

Keywords: lung cancer, systemic treatment, immunotherapy

1. Immunotherapeutic diagnosis

In order to have a therapy, it is known that we must first have a correct diagnosis. In this respect, we present an evolved oncology diagnostic system (http://www.carismolecularintelligence.com/i-o/). First of all, immunotherapy options should be sought through the development of complex immunoregulatory pathways. One of the systems that can be used in immunological diagnosis is Caris Molecular Intelligence. This system provides oncologists with reliable molecular information to make decisions about the use of immunotherapy. The tests are validated for testing PD-L1, MSI, and tumor mutation load (TML). Programmable cell death-ligand 1 (PD-L1) is one of the most important control immune proteins that mediates tumor-induced suppression by T-cell downregulation. Expression of PD-L1 may indicate a more likely response to immunotherapy. Of course, a perfect marker to predict the response

to PD-L1 inhibitor therapy has not been validated for the moment, but with these tests, we have an important orientation (Cochrane Collaboration Guidelines).

Microsatellite instability (MSI) is caused by the failure of the mismatch repair (MMR) system. MSI-High correlates with the increase in neoantigenic burden, which is more likely to respond favorably to immunotherapy.

Tumor mutation load (TML) measures the total number of non-sinusoidal somatic mutations identified on the megabase of the genome coding region. High TML supports neoantigens and responds favorably to immunotherapy.

2. Immuno-oncological agents: action mechanism

The immune system is capable of recognizing and destroying tumor cells as well as pathogens. However, one of the hallmarks of cancer is its ability to avoid the immune system [1].

There are a lot of complex interactions between the cells presenting the antigen, the lymphocytes, and the tumor cells. The most studied is the cell membrane T-cell receptor binding, called programmed cell death 1 (PD-1), and its ligands 1 or 2 (PD-L1 or PD-L2) expressed by some tumor cells. This interaction results in inactivation of T lymphocytes in an effort to avoid the immune response against tumor cells [2, 3]. Inhibition of this pathway is the target of inhibitors of immune control points. There are two types of agents: anti-PD-1 and anti-PD-L1 monoclonal antibodies.

Among these, anti-PD-1 agents that bind the lymphocyte receptor and block both PD-L1 and PD-L2 bindings are considered to be more toxic than anti-PD-L1 due to their broad spectrum of clinical activity. However, this has not been confirmed by recent clinical trials [4, 5]. Pembrolizumab and nivolumab, two monoclonal antibodies against PD-1, as well as avelumab monoclonal IgG1 anti-PD-L1 antibodies, atezolizumab and MEDI4736, showed consistent antitumor activity against NSCLC [6].

3. Lung cancer immunotherapy

Despite an improvement in overall survival (OS) by platinum-based chemotherapy (NSCLC Meta-analyses Collaborative Group, 2008), prognosis remains unsatisfactory for patients with advanced NSCLC, with a median survival of 8–12 months [7, 8].

In 2006, there was a plateau for chemotherapy in a study that none of the four chemotherapy regimens compared offered a significant advantage over the others in the treatment of advanced non-small cell lung cancer [8].

The development in molecular characterization of NSCLC, especially in histological subtypes of adenocarcinoma, has allowed the identification of key genetic aberrations in NSCLC, which can be addressed with molecular targeted therapy. Genetic aberrations in EGFR, ALK, ROS1, RET, BRAF, and NTRK have a predictive value for susceptibility to receptor tyrosine

kinase inhibitors [9–11]. Despite the success of molecular diagnostics, acquired resistance and disease progression are inevitable [9–11].

Treatment options for patients with small-cell lung cancer (SCLC) where the disease progressed after platinum-based chemotherapy are even more limited.

Immunotherapy in cancer has been described as any therapy that interacts with immunity. Immunotherapy in cancer can be classified into passive and active types. Passive immunotherapy has been described as administration of an active agent produced or generated outside the patient's body. Theoretically, such an approach does not depend on the host's own immune system to have an effect. Examples of passive immunotherapy include the use of monoclonal antibodies, such as trastuzumab [12, 13], and adoptive cell therapy, such as tumor-infiltrating lymphocytes (CAR-T cell) [14]. This new approach of therapy has and specific toxicity: cytokine release syndrome, neurologic toxicity, "on target/off tumor" recognition, and anaphylax [15].

Active immunotherapy involves stimulating or determining the host's immune system to recognize a tumor as a foreign. Examples of active immunotherapy include vaccination against cancer with tumor antigens and an adjuvant enhancement of immune cell function with cytokines, as well as targeting of immune control regulators with immune control inhibitor control.

Inhibitors targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) are used in NSCLC and SCLC.

Studies that examine the efficacy of cytokines such as interferon alpha and interleukin-2 (IL-2) in lung cancer patients were negative and will not be discussed [16].

3.1. Vaccines against cancer

Therapeutically acting vaccines in cancer are designed to eliminate cancer cells by increasing their own immune responses. This type of vaccine contrasts with prophylactic vaccines, which are usually administered to healthy people. Cancer vaccines can be classified into several major types, such as cellular vaccines, peptide vaccines, and genetic vaccines [17].

Vaccines against cancer, despite despite setbacks attempt to harness the patient's immune system to fight tumor cells and show a promise in clinical trials.

Cellular vaccines may be either autologous or allogeneic. Autologous tumor cell vaccines are developed by isolating tumor cells from an individual (patient), creating a vaccine that is administered back to the same patient, usually in combination with an adjuvant that stimulates the immune system. These vaccines have been among the first types of cancer vaccines tested and have the advantage of provoking an immune response to a wide range of tumors. Antigens expressed by the patient's own tumor result in tumor destruction. Although similar to autologous vaccines, allogeneic vaccines are obtained by administering tumor cells to a patient, creating a vaccine that is then administered to another patient with the same type of cancer [18].

Unlike cellular vaccines that are made directly from patient tumors, peptide vaccines are often synthesized in vitro to mimic tumor-associated proteins in order to elicit an immune response against tumor cells expressing that protein [19].

Genetic vaccines are composed of DNA molecules or synthetic RNAs encoding tumor-associated proteins and are administered either alone or packaged in a nonpathogenic virus. The genetic material is taken up by the recipient cells, translated into proteins encoded, processed, and presented to the immune system to elicit the immune response against tumor-associated proteins [20].

DNA vaccination has suddenly become a favored strategy for inducing immunity. The molecular precision offered by gene-based vaccines, together with the facility to include additional genes to direct and amplify immunity, has always been attractive. However, the apparent failure to translate operational success in preclinical models to the clinic, for reasons that are now rather obvious, reduced initial enthusiasm. Recently, novel delivery systems, especially electroporation, have overcome this translational block. Here, we assess the development, current performance, and potential of DNA vaccines for the treatment of cancer.

Early studies on Calmette-Guerin adjuvant Calmette-Guerin adjuvant and neoadjuvant bacillus vaccine therapy were negative [21, 22].

In the modern age, multiple-stage, locally advanced, and advanced NSCLC vaccine studies have been conducted. The recombinant protein-associated anti-melanoma-antigen-associated antigen (MAGE)-A3 vaccine has been extensively studied in adjuvant therapy after complete resection. A randomized phase II trial showed that for patients with stage IB–II, MAGE-A3 in NSCLC, who did not receive any adjuvant chemotherapy, there was a tendency toward survival gain. And, survival without signs of disease was positively influenced by the MAGE-A3 vaccine compared to placebo after a median follow-up to 70 months (HR, 0.75; 95% CI, 0.46–1.23; p = 0.254) [23].

However, clinical benefit was not found in the randomized, double-blind, placebo-controlled phase III (MAGRIT) study in fully resected NSCLC IB–IIIA MAGE-A3, with or without adjuvant chemotherapy. Subsequently, for the total population in this study, median disease-free survival was 60.5 months for the MAGE-A3 vaccine group and 57.9 months for the placebo group (HR, 1.02; 95% CI, 0.89–1.18; p = 0.74). In the subgroup that performed adjuvant chemotherapy, median disease-free survival was 58.0 months in the vaccine group and 56.9 months in the placebo group (HR, 0.97; 95% CI, 0.80–1.18; p = 0.76) [24].

Tecemotide (L-BLP25) is a peptide vaccine based on a 25 amino acid sequence of mucin-1 (MUC1), which has shown promising activity in locally advanced NSCLC in a phase II study [25].

Subsequently, the result led to the initiation of two randomized trials. One was a complete phase III trial, START, in which the placebo tecemotide was compared for patients with stage III NSCLC without disease progression after chemoradiation therapy [26].

The second study, INSPIRE, was a randomized phase II study of Asian patients that did not have convincing results after the Asian phase [27].

Analysis of the START study showed that there was no significant difference in median overall survival between the tecemotide arm and placebo arms (25.6 months vs. 22.3 months; HR adjusted, 0.88; 95% CI, 0.75–1.03; p = 0.123). However, following a prespecified subgroup

analysis, median overall survival was different between the vaccine arm and the placebo arm for patients receiving concomitant chemoradiation therapy (30.8 months vs. 20.6 months; HR, 0.78; 95% CI, 0.64–0.95; p = 0.016) compared with patients receiving sequential chemoradiation therapy (19.4 months vs. 24.6 months; HR, 1.12; 95% CI, 0.87–1.44; p = 0.38) [28].

In the advanced stage of the disease, the TG4010, another vaccine targeting MUC1, used a viral vector to express both MUC1 and IL-2 (a T-cell stimulus). The results were promising.

In a phase IIb study (TIME) results (part of the randomized, double-blind, placebo-controlled, phase IIb/III study), showed that in the overall population, disease-free survival was 5.9 months for the TG4010 group and 5.1 months for placebo (HR, 0.74; 95% CI, 0.55–0.98; p = 0.019) [29].

Belagenpumatucel-L is an allogeneic tumor cell tumor vaccine derived from four cell lines of NSCLC with different histologies, also express an antisense transgene for transforming beta2 growth factor that reduces the regulation of its immunosuppressive transformation. The results of a phase II study suggested clinical efficacy in patients with advanced NSCLC, and a randomized phase III (STOP) study was initiated. Patients with stage III/IV NSCLC in whom the disease did not progress after platinum-based chemotherapy received either belagenpumatucel-L or placebo [30]. There was no significant difference in overall survival between the two arms (20.3 months vs. 17.8 months; HR, 0.94; p = 0.594); there was also no difference in progression-free survival (PFS) (4.3 months vs. 4.0 months; HR, 0.99; p = 0.947) [30].

The epidermal growth factor receptor (EGFR) is an important signaling pathway in NSCLC, and a vaccine has been developed against its related EGF ligand, using recombinant human EGF coupled to a carrier protein. In a randomized phase II trial, patients with stage IIIB/IV NSCLC were randomly assigned to receive the best supportive treatment or EGF vaccines after first-line chemotherapy [31]. In the global population, there was a trend toward improved overall survival and a significant survival advantage for patients who had a good antibody response to the EGF [31].

A subsequent phase III study included patients with stage IIIB/IV NSCLC who were randomly assigned to the first line of chemotherapy to make the vaccine or the best supportive care. In the safety population, overall survival was 10.83 months for the vaccine arm and 8.86 months for the control arm [32]. For patients who received at least four doses of vaccine, overall survival differed significantly between the vaccine group and the supportive treatment group (12.43 months vs. 9.43 months; HR, 0.77; p = 0.036). In addition, overall survival was longer (14.66 months) for patients vaccinated with high concentrations of EGF at the baseline [32].

3.2. CTLA-4 inhibitors

Ipilimumab in combination with chemotherapy has been studied in patients with advanced NSCLC who have not received the previous treatment. In this phase II triple-arm study, patients were randomly assigned to chemotherapy (carboplatin plus paclitaxel), sequential chemotherapy with ipilimumab, or chemotherapy with concomitant ipilimumab. The primary endpoint of the study was overall survival and progression-free survival, which was 4.6 months for the chemotherapy arm, 5.7 months for the sequential ipilimumab chemo arm

(HR, 0.72; p = 0.05), and 5.5 months for the ipilimumab arm concomitantly with chemotherapy (HR, 0.81; p = 0.13) [33]. Progression-free survival was better in NSCLC patients with squamous histology than patients with nonsquamous NSCLC. To confirm these results, a larger phase III trial (NCT02279732) was initiated for patients with squamous cell NSCLC.

Conclusion of the study was that phased ipilimumab plus paclitaxel and carboplatin improved irPFS and PFS, which supports additional investigation of ipilimumab in NSCLC [33].

In the Govindan study ipilimumab added to chemotherapy (carboplatin plus paclitaxel) did not improve the survival of patients with advanced NSCLC [34].

3.3. PD-1 and PD-L1 inhibitors

PD-1 inhibitors include agents such as nivolumab and pembrolizumab. Nivolumab is a fully human immunoglobulin G4 (IgG4) monoclonal antibody that disrupts PD-1-mediated signaling, thus releasing T cells from their inhibitory interaction with PD-L1 and PD-L2. Pembrolizumab is a monoclonal antibody, the humanized IgG4/kappa isotype, which also blocks the binding of PD-L1 and PD-L2 to PD-1 on T cells, resulting in activation of tumor-specific cytotoxic T cells. Cytotoxicity is complement-dependent (CDC) (Alsaab) [35].

Action may be important because cytotoxicity can cause an exhaustion of activated T cells and infiltrating lymphocytes into tumors. PD-1 is expressed on effector T cells and other immune cells [36].

Checkmate 026 did not show a benefit in PFS for nivolumab versus chemotherapy. The authors reveal the fact that nivolumab monotherapy did not result in longer progression-free survival than platinum-based chemotherapy as first-line treatment for stage IV or recurrent NSCLC in a broad population of patients with a PD-L1 expression level of 5% or more. Overall survival with single-agent nivolumab was similar to overall survival with platinum-doublet chemotherapy. Nivolumab had a favorable safety profile as compared with chemotherapy, and no new safety signals were observed [37].

The new data from the phase 1b CA209-003 study were presented at the American Association for Cancer Research annual meeting: "The longest follow-up to date on patients treated with nivolumab for advanced non-small cell lung cancer (NSCLC) shows a 16% 5-year overall survival (OS) rate, according to new results presented here at the American Association for Cancer Research annual meeting." Suzanne Topalian, from Johns Hopkins University, and a coinvestigator (April 03, 2017): "the 5-year overall survival really quadrupled the survival that we would otherwise expect if these same patients had received chemotherapy" (April 03, 2017) (https://www.medscape.com/viewarticle/878148).

Nivolumab provides a long-term clinical benefit and a favorable tolerability profile compared to docetaxel in previously treated patients with advanced NSCLC [38]. FDA approved of nivolumab for second-line treatment of patients with advanced NSCLC.

In a single-arm phase II study (CheckMate 063) with nivolumab for patients with squamous cell NSCLC who were treated with third-line therapy and beyond, the partial response rate

was 14.5, and 26% of patients had a stable disease [4]. Overall survival was 8.2 months, and 1-year survival was about 41%. Noteworthy, the study population was very refractory to treatment, with 65% of patients treated with at least three previous systemic therapy lines. In addition, 61% of patients had disease progression as the best response to the latest therapy [39].

In another phase II trial (CheckMate 153), 824 patients with advanced NSCLC were treated for 1 year with nivolumab. The partial response and stable disease rates were 12 and 44%, respectively. The answers were independent of the PD-L1 expression [40].

The second-line treatment with nivolumab was superior to docetaxel in two subsequent phase III randomized phases in advanced NSCLC patients receiving double-blind platinum chemotherapy.

In a study of 272 patients with squamous NSCLC (CheckMate 017), median overall survival and 1-year survival were better for nivolumab than for docetaxel. The risk for death was 0.59 with nivolumab ($p < 0.001$) [6].

In the study (CheckMate 057), which included patients with advanced nonsquamous NSCLC histology, nivolumab in line 2 was also associated with better overall survival and survival over 1 year, also better than docetaxel (HR, 0.73) [41]. In subset analysis of subset biomarker values, PD-L1 expression ≥1, ≥5, and ≥10% corresponded to an improvement in PFS with a HR of 0.70, 0.54, and 0.52, respectively, and in OS with a HR of 0.58, 0.43, and 0.40. In contrast, in tumors with a low PD <1, <5, and <10% PD-L1 expression, HR for PFS was 1.19, 1.31, and 1.24, respectively, and for OS was 0.87, 0.96, and 0.96 [41].

The safety and efficacy of single-agent nivolumab in first-line treatment of patients with advanced NSCLC have been reported in CheckMate 012 adverse events occurred in 71% of patients, the most common being fatigue (29%), rash (19%), nausea (14%), diarrhea (12%), pruritus (12%), and arthralgia (10%). The overall confirmed response was 23%, and progression-free survival and overall survival were 3.6 months and 19.4 months. The nonprogression-free survival rate of 24 weeks was 41%. The survival rate at 1 year was 73% [42].

Recently, in a phase III study, first-line nivolumab compared to a platinum-based chemo-therapy for tumors with a PD-L1 expression of 5% or greater (CheckMate 026) showed progression-free survival greater for the chemotherapy arm, but overall survival was better for the nivolumab arm [43]. The objective response rate was lower for the nivolumab arm. In conclusion, nivolumab monotherapy did not result in longer progression-free survival than platinum-based chemotherapy as first-line treatment for stage IV or recurrent NSCLC. In this study the PD-L1 expression level was 5% or more [43].

3.4. Activity in SCLC

SCLC is most often an extended stage disease at the time of diagnosis. Although the first line of platinum-based chemotherapy has activity, the disease progresses inevitably, and response rates in the second-line treatment are low and are not sustainable. The activity and safety of nivolumab

with or without ipilimumab in previously treated SCLCs were evaluated in CheckMate 032. The objective response rate was 10% with nivolumab 3 mg/kg alone, 23% with 1 mg/kg of nivolumab in combination with 3 mg/kg of ipilimumab, and 19% with 3 mg/kg of nivolumab in combination with 1 mg/kg of ipilimumab. PD-L1 expression was not associated with responses [44].

Patients with small-cell lung cancer (SCLC) and a high tumor mutation burden had an important increase in survival (near doubling in response rate and 1-year overall survival) with ipilimumab combined with nivolumab versus nivolumab alone.

The efficacy and safety of pembrolizumab at two different doses in previously untreated patients, advanced NSCLC, were reported in the Keynote-001 study. The objective response rate was 19.4%, and the median response time was 12.5 months. The progression-free survival was 3.7 months, and overall survival was 12.0 months [45]. The objective response rate was 18% in those treated previously and 24.8% of untreated patients. The objective response rate was 45.2%, and no time to progression was 6.3 months. The objective response rate was similar regardless of dose, schedule, and histology subtype. The response rate was higher among smokers than nonsmokers. Treatment-related adverse events of any grade occurred in 70.9% of patients, 9.5% having a grade 3 or higher adverse event [45].

Pembrolizumab was evaluated in a phase II/III study of patients previously treated with advanced NSCLC (Keynote-010). A total of 1034 patients were randomized to receive either 2 mg/kg dose or 10 mg/kg of pembrolizumab or 75 mg/m² of docetaxel every 3 weeks [46]. All patients had at least 1% tumor cells that were positive for PD-L1. Overall survival was improved with both doses of pembrolizumab compared to docetaxel. Among patients with at least 50% of the tumor cells expressing PD-L1, overall survival rates were 14.9 and 17.3 months with pembrolizumab at doses of 2 mg/kg and 10 mg/kg, respectively, compared to 8.2 months with docetaxel. Any degree of treatment-related adverse events occurred in 63% of pembrolizumab 2 mg/kg and 66% of patients receiving 10 mg/kg. The treatment-related toxicity was higher (81%) in the docetaxel arm.

Grade 3–5 treatment-related adverse events were less common in pembrolizumab-treated patients (2 mg/kg (13%), 10 mg/kg (16%)) versus docetaxel (35%) [46].

The Keynote-024 phase 3 clinical trial was the basis for pembrolizumab approval as a first-line treatment for patients with a diagnosis of metastatic NSCLC for whom PD-L1 expression is in 50% or more of tumor cells. Keynote-024 is a randomized, open-label phase 3 study evaluating pembrolizumab monotherapy at a fixed dose of 200 mg compared to the platinum-based chemotherapy standard for the treatment of patients with metastatic NSCLC with both squamous and unscrupulous histologies.

In phase III trial for first-line therapy of patients with advanced NSCLC (Keynote-024), with a PD-L1 tumor expression of 50% or greater, patients were randomly assigned to pembrolizumab- or platinum-based chemotherapy doublets, and progression-free survival was significantly better for pembrolizumab (HR, 0.50, 95% CI, 0.37–0.68; p < 0.001) median 10.4 months [47].

Overall survival was 0.60 (95% CI, 0.41–0.89; p = 0.005). The estimated percentage of patients in life at 12 months with pembrolizumab was 70%. In addition, the response rate was higher for pembrolizumab than for chemotherapy. Adverse events associated with pembrolizumab

therapy were fewer than chemotherapy. The results are innovative because this is the first to demonstrate the superiority of anti-PD-1 therapy to platinum [47].

3.5. Activity in SCLC

Preliminary data from a multicohort phase Ib study on pembrolizumab with previously treated PD-L1-positive subjects include a 25% objective response rate and a 31% disease control rate [48].

3.6. PD-L1 inhibitors

3.6.1. Avelumab and atezolizumab

PD-L1 inhibitors also inhibit PD-1/PD-L1 interactions. PD-L1 inhibitors include atezolizumab, durvalumab, and avelumab. Atezolizumab and durvalumab are human IgG1 anti-PD-L1 antibodies with mutations in their Fc domains to eliminate both antibody-dependent cell-mediated cytotoxicity (ADCC) activity and complement-dependent cytotoxicity (CDC) activity. Avelumab is a fully human IgG1 anti-PD-L1 monoclonal antibody and, unlike another PD-1/PD-L1 inhibitor, has been shown to retain ADCC and CDC activity in preclinical studies [49].

In a single-arm phase II study (IMpower 110 study), the objective response rate for atezolizumab was 16%, regardless of PD-L1 expression in immune cells, and 28% in patients with 5% or more high expression PD-L1 [50]. Atezolizumab (MDPL3280A) clearly is an added value in the treatment of advanced-stage pretreated NSCLC. Its interest in contrast with other immune checkpoint inhibitors relies on its efficacy, even in low or no PD-L1 expression subgroups. Considering that the efficacy of anti-PD-1 such as pembrolizumab or nivolumab is overall higher in PD-L1-positive patients, atezolizumab might be preferable in PD-L1-negative patients. It will be necessary to consider other variant methods of PD-L1 testing used for each therapy to further explore this hypothesis [51].

In a randomized phase II (Poplar) study in patients receiving platinum-based chemotherapy, atezolizumab was associated with a higher overall survival (HR, 0.73; CI 95%, 0.53–0.99; p = 0.04) [52]. In another phase II trial (BIRCH), advanced NSCLC patients who were selected for PD-L1 expression received atezolizumab as first-line or as a subsequent therapy. Response rates ranged from 17 to 27% [53], and median overall survival was 14 months for patients receiving atezolizumab as the first line of therapy. Overall survival has not yet been achieved for patients receiving atezolizumab as a subsequent therapy [53]. In the OAK study, a phase III trial of previously treated NSCLC patients randomly assigned to atezolizumab or docetaxel, the overall survival was significantly better for atezolizumab (13.8 months vs. 9.6 months; HR, 0.73; 95% CI, 0.62–0.87; p = 0.0003) [54]. The OAK study led to the FDA approval of atezolizumab for second-line therapy of advanced NSCLC [54].

3.6.2. Durvalumab

In a phase I/II study with durvalumab in 2009 in the first-line treatment in NSCLC patients irrespective of PD-L1 status, the overall response rate was 27 and 29% for PD-L1-positive tumors (defined as ≥25% of tumor cells expressing PD-L1) and 11% in PD-L1-negative tumors [55]. In a phase II trial of patients with advanced NSCLC who received at least two previous systemic therapy lines, the activity was extremely encouraging. The objective response rate

and survival rate at 1 year increased according to the PD-L1 expression: 7.5% (PD-L1 expression less than 25%), 16.4% (more than 25% expression), and 30.9% (greater than 90% expression). The corresponding 1-year survival rates were 34.5, 47.7, and 50.8% [56].

The study PACIFIC was presented to the ESMO Congress 2017 and was a randomized, double-blind, international, phase 3 study comparing durvalumab as consolidation therapy with placebo in patients with stage III, locally advanced, unresectable NSCLC that had not progressed after platinum-based chemoradiotherapy. Median progression-free survival as assessed by means of blinded independent central review was 16.8 months (95% confidence interval [CI], 13.0–18.1) with durvalumab versus 5.6 months (95% CI, 4.6–7.8) with placebo (stratified hazard ratio for disease progression or death, 0.52; 95% CI, 0.42–0.65; two-sided $p < 0.001$). Authors consider that this study will change the clinical practice [57].

3.7. Combinations of immunotherapy agents

CTLA-4 and PD-1/PD-L1 are combination. CTLA-4 inhibitors are also studied in conjunction with PD-1 and PD-L1 inhibitors. Results of preclinical studies indicate that this combination can work synergistically to produce improved antitumor activity [58].

Nivolumab was combined with ipilimumab for first-stage NSCLC in setting up in a phase I (CheckMate 12) study. The results included objective response rates ranging from 13 to 39%.

In NSCLC, the first-line nivolumab plus ipilimumab had a tolerable safety profile and showed an encouraging clinical activity characterized by a high response rate and durable response. In our study, the results of this study are the first suggestion of improved benefit compared with anti-PD-1 monotherapy in patients with NSCLC, supporting further evaluation of this combination in a phase 3 study [59].

Durvalumab was combined with the tremelimumab CTLA-4 inhibitor in a phase Ib study of patients with advanced NSCLC. Although many adverse events occurred during the study dose phase, the antitumor activity (23% objective response rate) was evident regardless of the PD-L1 status in the evaluable patients in the dose study—the expansion phase of the study [60].

In a phase III randomized study, the frontline durvalumab, either in combination with tremelimumab or as a single agent, did not improve progression-free survival (PFS) in patients with stage IV metastatic non-small cell lung cancer (NSCLC) compared with standard platinum-based chemotherapy [61].

4. Conclusions

Immunotherapy has become one of the most important therapeutic tools in advanced lung cancer. Existing studies have revealed a response rate of between 13 and 39%. It is also important that this therapy, unlike TKI-targeted therapy, also responds to smokers who make up most of the lung cancer patients.

Another important benefit from immunotherapy in advanced lung cancer is that squamous non-small cell lung cancer also responds to this therapy. Some promising results are and in treatment of small-cell lung cancer.

From existing studies, it is trembling that immunotherapy can improve survival compared to chemotherapy in a selected patient population, both in the first line and in the second line.

There is not yet a valid predictive marker that can be used to choose patients who will respond to immunotherapy. Currently, the only marker used is PD-1 expression that does not have a good validity. For the moment, there are not criteria to select patients for treatment with PD-1 or PD-L1 inhibitor because data to compare these two pathways is lacking. Better results were however obtained with a percent of PD-L1more then 50%. More study are needed to define the best combination of immunotherapy with chemotherapy or radiotherapy.

Vaccine therapy is promising but needs additional evaluation. Vaccine in combination with other therapeutic modalities especially checkpoint inhibitors is possible to have some benefits and must be studied.

Many guidelines are developed to treat side effects of immunotherapy. Despite a correct supportive therapy, some side effects are life-threatening. But generally, the quality of life of patients treated with immunotherapy is improved.

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Epigenetic Modifications and Potential Treatment Approaches in Lung Cancers

Metin Budak and Mustafa Yildiz

Additional information is available at the end of the chapter

Abstract

Alteration of methylation is a process seen across a wide variety of species ranging from bacterial microorganisms to mammals, defined as the adaptation method the organism develops against environmental or intrinsic effects, or employs to switch off the genome regions which are no longer required through the evolutionary process. Scientific advancements have allowed detecting the regions that undergo different patterns of methylation. It has been demonstrated that the control on changes in gene expression is not guided by transcription factors alone and that epigenetic alterations are also involved in this process. Furthermore, epigenetic modifications have been shown to be considerably important in cancer development. This section focuses on epigenetic changes and potential treatment options in lung cancer.

Keywords: lung cancer, epigenetic, acetylation, methylation, treatment

1. Introduction

Lung cancer is one of the leading causes of death worldwide. The five-year survival rate is approximately 15% as the condition is often diagnosed at an advanced stage. Smoking is the underlying cause of about 90% of all lung cancers, and smokers constitute the major risk group for developing lung cancer. World Health Organisation (WHO) stratifies lung cancers into two histological groups: non-small cell lung cancer (NSCLC), accounting for 85% of the cases, and small cell lung cancer (SCLC), which constitutes the remaining 15%. The most valid hypothesis for the development of these cancers suggests that multi-phase genetic alterations and a series of epigenetic events result in lung cancer [1].

Owing to the technological advances of the twenty-first century, current practices offer personalised treatment alternatives by means of detailed diagnostics and a range of treatment approaches in lung cancer. While World Health Organisation classified lung cancers almost entirely under the category of adenomas until 1960s, the utility of advanced molecular tests has allowed convenient and thorough identification of subtypes. In addition with these developments, new molecular targets have emerged, leading to novel therapies [2]. During the last 20 years, studies on molecular mechanisms seeking to elucidate the development of cancers and the underlying mechanisms have shown the importance of epigenetic changes in these processes, leading to increasing interest and studies in this field.

Like all cancers, lung cancer develops with the deviation from a normal cell structure due to a number of problems that arise during cell cycle and differentiation. Deviations from normal state to tumour formation result from alterations in cell growth-signalling pathways and apoptosis mechanisms, including a series of epigenetic modifications, all of which are critical for the cell [3].

Epigenetic modifications usually occur in two forms: (1) acetylation, which occurs mainly in histone proteins at protein level and (2) methylation, which is often seen at DNA level.

2. Mutations in lung cancer

Small cell lung cancer (SCLC) accounts for approximately 16–20% of all lung cancers. Owing to the rapidly spreading pattern, SCLC is accepted as an aggressive and widespread disease; therefore, chemotherapy (CT) remains an important modality for the treatment of SCLC.

Non-small cell lung cancer (NSCLC) is responsible for about 85% of lung cancers. Postoperative adjuvant treatment approaches have become the standard of care in early stage tumours, and various systemic treatments are utilised in a metastatic setting. Although the systemic treatment approach in NSCLC according to the subtype has not changed profoundly through the years, the selection of a systemic treatment in metastatic cases has recently begun to differ based on molecular alterations and different histological subtypes of NSCLC. In addition to molecular changes that provide predictions for targeted therapies, separating NSCLC into two groups, that is, squamous and non-squamous, has been suggested to aid in selecting a more effective chemotherapy agent [4–6].

Lung cancer can be histologically divided into two main groups as NSCLC and SCLC. Mutations may be seen in genes such as EGFR, RET, PIK3CA, ALK, HER2, KRAS, BRAF, MET, NRS, MEK1 and ROS1, which are often referred to as signalling pathways and considered as drivers since they cause cancer development in NSCLCs. These mutations can be seen in current smokers, ex-smokers and non-smokers, whereas mutations in EGFR, ALK, HER2, ROS1 and RET genes are seen only in people who have never smoked. Such mutations may be observed in all histological subgroups of NSCLCs including adenocarcinomas, squamous cell carcinomas (SCCs) and large cell carcinomas [7–10].

Smoking causes chronic inflammatory stress on biological systems, thereby interfering with the cell cycle, cell development and differentiation. Long-term inflammation is associated with DNA methylation and contributes to lung cancer development via methylation mechanisms.

For example, genes such as APC, FHIT, RASSF1A and CCND2 are inactivated only in smokers due to promoter hypermethylation. Increased promoter hypermethylation of P161NK4A, MGMT, RASSF1A, FHIT and MTHFR, depending on the intensity of smoking, shows a strong correlation with NSCLCs compared to non-smokers [11, 12]. Promoter methylation of RARβ, FHIT, P161NK4A and RASSF1A increases as smoking intensifies.

Moreover, mutations in epigenetic regulator genes create a complicated situation owing to the fact that they prevent these genes from functioning properly, which is expected to impact cell cycle and cell development, thereby resulting in the development of cancer. However, these mechanisms may also offer certain advantages in favour of treatment as they may open new ways for cancer therapies (such as DNTM inhibitors) [13].

3. Acetylation of histones

Human genome, as that of any eukaryotic organism, is organised in a highly complicated manner. Except for the alterations in the genes effective in human development, the histone proteins that pack the genome serve to control the genome by undergoing various modifications. This is accomplished through various changes that occur during events such as DNA replication, repair and expression [14].

For instance, the amino terminal of the histone core is quite important as it contains a flexible and fairly simple tail domain, constituting a target region for several post-translational modifications. Histone modifications primarily occur in the form of addition of acetyl and methyl groups to lysine amino acids, addition of phosphorus to serine amino acids and methyl groups to arginines. These modifications play a critical role in the control of biological processes such as transcription [15].

Modifications in histone proteins develop by means of enzymatic pathways. Histone deacetylase inhibitors (HDACIs) also act as antagonists in cell differentiation by acetylating histones and non-histone proteins. Proteins in this group inhibit DNA repair, apoptosis and gene expression mechanisms. In addition, such proteins contain a zinc-binding group (ZBG) and a chain linking two proteins from these two groups referred to as the surface recognition polypeptide [16]. Therefore, molecules able to inhibit such proteins may be potential anti-cancer agents. For example, peptide-containing cyclic hydroxamic acids (CHAPs) may be suitable for therapeutic use as a group of potential anti-cancer agents. The molecule CHAP31, which acts on HDACIs, has been shown to have a highly effective anti-tumour effect on certain types of cancer such as lung, breast and gastric cancer as well as melanoma *in vivo* [17].

Fibrosis is one of the important factors contributing to cancer invasion and metastasis. Fibrosis results from fibroblast activation, which degrades and alters the physical structure of extracellular matrix (ECM). The fibrosis-induced increase in ECM fragility leads to pathological conditions such as epithelial-mesenchymal transition (EMT) with the transformation of normal cells into cancer. The change in the structure of collagen, one of the most important proteins in tissue structure, is another contributing factor. For this reason, collagen receptors are of particular importance regarding the progression of cancer. The discoidin domain receptors

(DDRs), DDR1 and DDR2, are overexpressed receptor proteins. DDRs mechanically increase the acetylation of c-Myb, the transcription factor of histone acetyltransferase (HAT) on rigid ECM, thereby leading to c-Myb binding to DDR2 promoter together with LEF1, and result in DDR2 upregulation in a rigid environment. Silenced c-Myb may cause DDR2 inhibition and invasion of lung cancer cells, and recovery of physical characteristics of the tissue has been shown when external interventions allow DDR2 expression [18].

Proteins in the Snail group, a zinc-binding superfamily of transcription factors, are responsible for cell migration and invasion during both the embryonic period and cancer process [19]. Snail proteins bind to the E-box sequence located in the promoter region of E-cadherin gene, which encodes the protein that is responsible for cell–cell adhesion. As a result, since E-cadherin synthesis is no longer possible, cell–cell adhesion cannot be achieved, and cancer cells gain metastatic properties [20]. Snail proteins are overexpressed in several types of cancer as they are strategically important for cancer cells. Rui et al. have emphasised that Snail, acetylated by P300, may be of value in terms of developing personalised treatments for lung cancer [21].

Furthermore, HDACs allow E-cadherin expression through non-coding RNAs. In addition, miRNAs serve to control EMT in lung cancer via TGF-β-, EGF- and HGF-signalling pathways. MiR200b and miR200c are effective on H3 acetylation in E-cadherin promoter (**Figure 1)** [22].

Epigenetic readers recognise modified histones by means of a group of polypeptides referred to as 'reader', and these polypeptides are involved both in normal cell growth and in cancer development by controlling several processes conducted together with chromatin [23].

Recently, Wenyi mi et al. identified the YEATS domain, defined as a novel acetyl-lysine-binding module. With regard to human cancers, the functional importance of proteins containing this domain remains unknown; however, the overexpression of the YEATS2 gene has been shown in non-small cell lung cancers. This domain is thought to decrease histone acetylation, thereby inactivating key genes [24, 25].
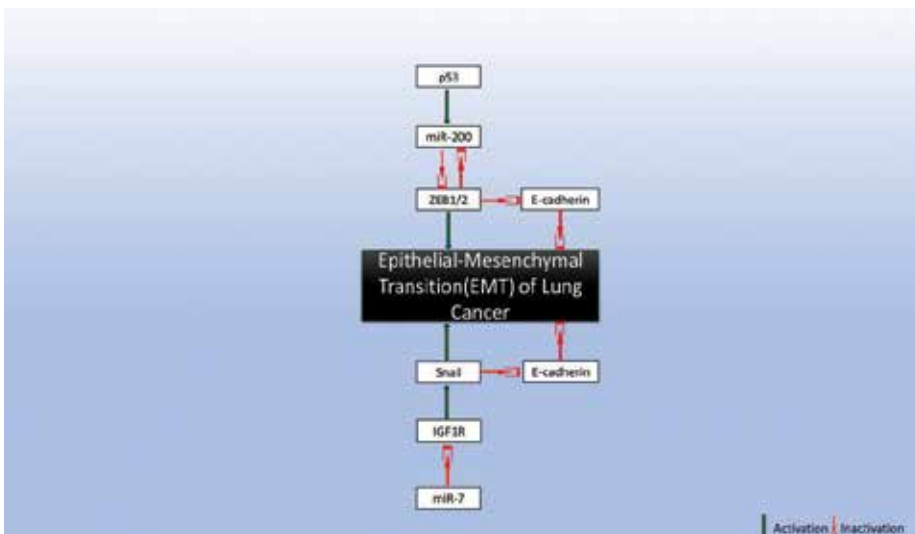

Figure 1. IGF and p53 pathways in lung cancer.

'Lunasin' is a soy protein consisting of 43 amino acids known to protect mammals against chemical carcinogens. In addition, it has been shown to be potentially beneficial in several conditions [26, 27]. A derivative of this protein originating from wheat has been reported to be effective even when taken orally and potentially effective in cancer prevention, regardless of the suppressed acetylation of core H3-H4 histone proteins [28].

As is the case in any intracellular event, the acetylation process occurs by means of enzymatic pathways. The removal of acetyl groups from histones is conducted by enzymes called histone deacetylases (HDACs). Cell viability continues normally as this is carried out in a balance of acetylation-deacetylation processes [29, 30]. Sometimes, hypoacetylation may occur when the process shifts towards deacetylation, and this is accompanied by cancer development. HDAC inhibitors are thought to possess the potential to reverse this process, leading to epigenetic reactivation of the suppressed anti-tumour genes [30]. This may help the suppression of certain tumours depending on which genes are expressed and which proteins are suitable.

Long Chen et al. believe that lysine acetyltransferase accelerates tumour formation due to the acetylation of histones and non-histone proteins despite the anti-tumour effect provided by acetylation-related HDAC inhibitors, and suggest that acetylation may shift to both sides (acetylation-deacetylation) depending on which genes are active at the time [30]. They highlighted the necessity to elucidate the relationship between lysine acetyltransferases (KAT) or HDAC and other proteins such as transcription factors in order to enhance the specificity.

Human MOF (hMOF and MYST1) is a member of the histone acetyltransferase (HAT) protein family. These proteins convert histone H4 acetylation to H4K16Ac, an epigenetic marker of active genes, in particular by adding an acetyl group to the lysine-16 amino acid. Irregularities in these epigenetic markers affect cell biology, potentially leading to cancer development. In correlation with H4K16Ac, hMOF has been shown to be overexpressed in non-small cell lung cancers. Investigators have indicated that this group of proteins may have potential oncological tasks and this may be a potential therapeutic target [31].

Although the interferon regulatory factor 3 (IRF-3), an important transcription factor for interferon genes, is often functional in viral infections, the regulation mechanism of IRF-3 expression in cancers has not been fully understood. The concurrent use of histone deacetylase inhibitors and Trichostatin A (TSA) has been shown to increase IRF-3 expression in lung adenocarcinoma A549 cells by altering GATA-1 acetylation, and targeting IRF-3 is therefore thought to be a novel therapeutic approach [32].

In light of all this information, one may conclude that some genes or proteins contribute to carcinogenesis when they function towards acetylation, while others contribute to carcinogenesis when they function towards deacetylation [22].

4. Methylation

In living organisms, all molecular structures and events are generated by specific sequences called genes that are found in the DNA molecule. However, these genes need to be governed and controlled so that they can participate in biological processes at the optimal time. Genes are actively controlled by specific genes and proteins referred to as transcription factors. However, there is a

different mechanism that also determines gene expression, which can be transmitted from generation to generation and from cell to cell. This is called the epigenetic code [12]. DNA sequence does not undergo any changes during the formation of this code, but the relevant part of the DNA fragment becomes no longer meaningful. The most common epigenetic modifications are the changes in histone proteins and DNA methylation. The most widely studied and the most well-established epigenetic mechanism is DNA methylation. It is an enzymatic change where cytosines are converted to 5′-methylcytosine. The cytosine-end methylation seen in mammalian genome often occurs at the nucleotide pairs which are also called the CpG dinucleotides. Detection of these methylated gene segments on the genome is highly informative in terms of the effects of genes in several biological processes from carcinogenesis to ageing [33, 34].

Methylation-related changes may occur anywhere in the genetic material of a eukaryotic cell. In eukaryotic cells, genetic material is found in two organelles: the nucleus, which contains almost all of the genetic material, and mitochondrion, which has a very small genome compared to the nucleus. Certain methylations are specific to the genomes of these two organelles [1].

For several years since being discovered, the pattern of DNA hypomethylation in CpG dinucleotides has been shown to be highly important in cancer cells [35, 36].

A methylated gene becomes inactivated and therefore cannot synthesise the product, that is, the RNA or protein. Gene methylation occurs more commonly in promoter regions. Methylation in the promoters of tumour suppressor genes is mostly associated with carcinogenesis and often occurs in the form of hypermethylation [12].

Apart from global DNA hypomethylation reported in earlier stages, hypomethylation may also occur in the CpG islands in promoter regions of several specific genes or in the nucleotide pairs of genes that are activated by hypomethylation and silenced by hypermethylation. Methylation of a promoter region does not necessarily produce any protein or RNA [37]. The SHOX2 gene, a member of the Homeobox gene family, has been shown to be a specific biomarker with 60% sensitivity and 90% specificity when investigated by bisulphite modification-PCR in blood plasma samples during a case–control study of approximately 400 subjects with lung cancer. Also, in the same study, comparison against the results from another study conducted with bronchial aspiration samples revealed a higher level of sensitivity compared to results from blood plasma samples [38]. In bronchoalveolar lavage samples obtained from NSCLC patients, 24% methylation was observed in the promoter region of the CDKN2A gene, also known as P16INK4A, in addition to microsatellite instabilities and p53 mutations [39, 40].

The lungs are highly exposed to external factors owing to the nature of their functions. This constitutes a major risk factor in terms of epigenetic modifications as well as being associated with pulmonary diseases caused by environmental factors and smoking in particular. DNA methylation, histone modification and non-coding RNA have been shown to be increased in smokers [11].

Lung cancer develops upon the accumulation of numerous genetic and epigenetic alterations in the respiratory epithelium. Early promoter methylation and tumour suppressor gene inactivation are considered as signs of pulmonary carcinogenesis [12].

Defects in the apoptotic pathway are among the main reasons contributing to the high fatality of lung cancers. Apoptosis, also known as programmed cell death, has a wide range of physiological

effects from embryonic stages to tumour formation. Inhibition of apoptosis is particularly detrimental in cancer treatments. The main reason of this is the fact that most treatments exert their effects by activating apoptotic mechanisms. Targeting the apoptotic pathway ensures the effectiveness of anti-cancer treatments [41, 42].

Survivin, one of the apoptosis inhibitor proteins, plays a critical role in cell division and in the continuation of cell survival [43, 44]. Since increased expression of survivin in human tumours leads to aggressive tumour development and resistance to main cancer treatments such as chemotherapy and radiotherapy, survivin gene and protein have been found to be important markers regarding the outcome of treatment [45, 46].

Computer analyses have shown a potential methylation region in exon 1 of the survivin gene; however, no methylation was found in lung cancer patients, and it has been shown that survivin gene expression in any cell may be effective not only with methylation but also through other transcription factors [47].

5. Importance of apoptosis

Apoptosis occurs during the normal development of multicellular organisms and continues throughout life. This mechanism is responsible for embryonic development and organ formation through cell differentiation. For example, toes are separated from one another by means of apoptosis.

Apoptosis also controls the immune system. T-lymphocytes are involved in the destruction of infected or damaged cells during cellular immunity. The T-lymphocytes produced in the thymus gland need to be active against foreign antigens before being released into bloodstream and should not show any activity against normal cells. Any inactive or semi-active T-cell is bound to be destroyed by apoptosis before they may begin their task.

Inhibitors of apoptosis proteins from the anti-apoptotic protein family have been identified in vertebrate and invertebrate species, and they are known to be negative regulators of programmed cell death. Some homologues identified in mammals include XIAP, cIAP1, cIAP2, NAIP, Bruce, Survivin and IAP. Most of these block cell death by directly binding to and inhibiting caspase-3, caspase-7 and caspase-9 [32].

Various diseases may arise in the event of any defect within the regulation of apoptosis. Among these, cancer is a condition characterised by little or no apoptosis. The mutations in cancer cells result in different cell-signalling and cell growth processes compared to normal cells. Under normal circumstances, when cells become damaged, they become apoptotic while cancer cells do not undergo apoptosis as a result of the cancerous mutations, which leads to uncontrolled cell differentiation and tumour formation. It is often difficult to eliminate such tumours with cell-damaging treatments such as chemotherapy and radiotherapy. In addition, some cancer cells develop resistance to treatments that target tumours with mutations in apoptotic pathways. Further understanding of the regulation of apoptosis in cancer cells is expected which allow developing novel therapies. While apoptosis is reduced in cancer, conditions with increased apoptosis lead to different problems. For example, neurodegenerative diseases such as Alzheimer's and Parkinson may be the result of increased cell death [43–46, 48].

6. Survivin and apoptosis

In early studies, caspase-3 suppression was suggested to be directly responsible for the anti-apoptotic mechanism of action of survivin. However, three-dimensional structural studies have shown that BIR (baculovirus IAP repeat domain) of survivin is not long enough to block the enzymatically active region of caspase-3. Survivin is thought to directly bind to caspase-9 as three-dimensional studies have revealed the similarity between the BIR domain of survivin and BIR domain of another IAP, XIAP, which directly binds to and inhibits caspase-9 *in vitro*. Another relevant mechanism is the inactivation of a pro-apoptotic molecule called SMAC/Diablo released together with cytochrome-c during mitochondrial apoptosis. SMAC/Diablo binds to IAPs and prevents their caspase-suppressing effect. Theoretically, survivin binds to SMAC/Diablo. Survivin bound to SMAC/Diablo protects other IAPs from the inhibitory effect of this protein. Thus, caspase suppression continues, leading to apoptosis blockade. There is evidence indicating that survivin plays an important role in p53-associated apoptosis. p53 blocks survivin transcription both through direct and indirect pathways. Conversely, overexpression of survivin inhibits p53-dependent apoptosis.

Phosphorylation of threonine-34 residues is necessary for survivin to bind to caspase-9. This is conducted by a kinase called p34cdc2-cyclin B1. Survivin-caspase interaction is shown in **Figure 2** [38].
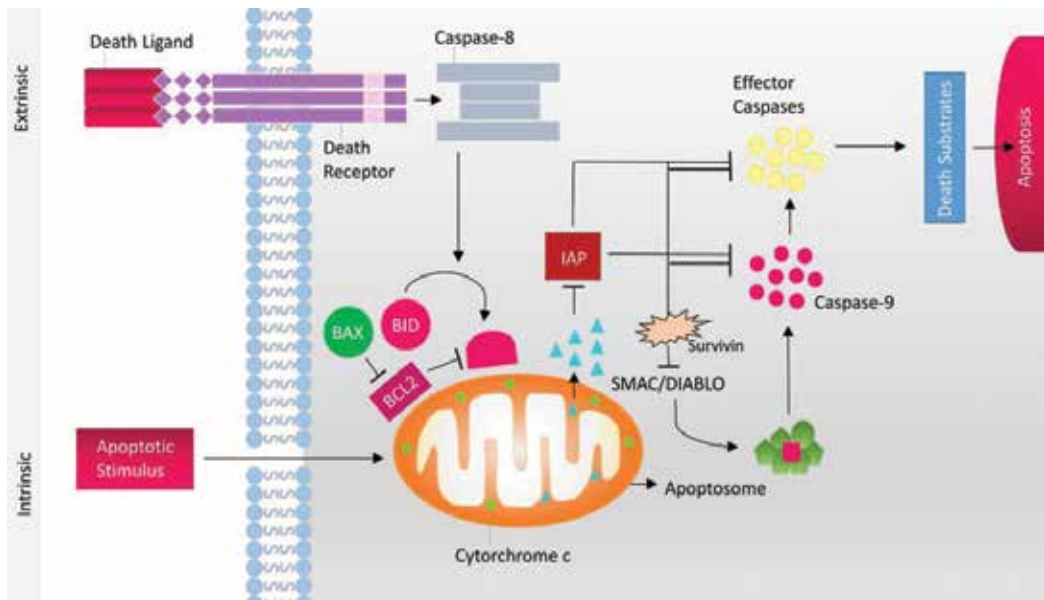

Figure 2. Apoptotic pathways.

7. Survivin gene and protein

Survivin molecule is expressed in special tissues and cells during certain phases of cell cycle. In humans, survivin expression occurs in the heart, liver, gastrointestinal tract and other foetal tissues from embryonic development until the end of foetal period, and in stem cells, epithelial cells and pancreatic endocrine cells during adulthood. Increased survivin expression has been shown in solid tumour tissues of adults, for example, lung, breast, brain, stomach, oesophagus, pancreas, liver, uterus and ovarian tumours. Increased survivin expression has also been reported in certain tumour tissues such as neuroblastoma, colorectal cancer and gastric cancer and has been associated with poor prognosis in these patients [49, 50]. Some studies have indicated the promotion of tumour development via survivin overexpression. There are numerous studies showing that survivin plays an important role not only in cell division and inhibition of apoptosis but also in cancer development [50, 51].

The human survivin gene is located on the telomeric position of chromosome 17 with a size of 14.7 kb. Survivin gene consists of four exons and three introns and encodes the survivin protein of 16.5 kD. While other IAPs contain more than one BIR in their structure, the survivin gene contains only one BIR region at the N-terminal domain and also contains an alpha-helix structure at the C-terminal domain. Survivin protein interacts with caspase-7 and caspase-9 via the BIR region while its alpha-helix structure interacts with tubulin subunits during mitosis [52, 53].

Survivin expression is suppressed during the G1 and S phases of the cell cycle but increases during G2/M. This control mechanism mostly functions at transcription level, occurring through cell cycle-dependent elements (CDEs) and cell cycle homology regions (CHRs). The CDE/CHR suppressor protein binds to this region, thereby suppressing gene expression. The CHR region is located at the proximal end of the survivin gene promoter [52, 53].

Cell-culture studies have demonstrated the association between SurKex methylation region and histone acetylation in the promoter region of the survivin gene. These studies have also reported a decreased mRNA expression in the survivin gene with the methylation of survivin promoter [54].

The methylated survivin promoter region has also been shown to inhibit p53 binding, which may render p53 ineffective in cell cycle [55].

For this reason, the studies on survivin gene region and protein have been intensified, with a steady increase in the number of studies investigating the expression analysis of survivin as well as survivin promoter-related polymorphisms and mutations.

8. Mitochondrial methylation

Mitochondrial functions provide several protein components synthesised from mitochondrial DNA (mtDNA) for the oxidative phosphorylation mechanism. In mammals, 12S and 16S

rRNAs are encoded with 13 proteins from mtDNA. These genes are effective on cell homeostasis and apoptotic pathways [56].

Since mitochondria are dominant organelles regarding intracellular energy and because they have their own independent DNA genome, it is important to look at the genomic alterations of this organelle in certain diseases. These alterations are associated particularly with various pulmonary diseases and lung cancers [57, 58].

As mentioned earlier, mitochondrial methylation may also be affected in lung cells, which are considerably exposed to environmental effects. In their study dated 2013, Byun et al. have shown that mitochondrial RNA may undergo methylation [59].

9. Mechanisms of current therapies in lung cancer

Several treatment modalities are utilised in different subtypes and stages of lung cancer. Surgery is the first-choice treatment if tumour margins are well defined while adjuvant or neoadjuvant chemotherapy and hormone therapies are also applied with or after surgery. Treatment often continues in the form of chemotherapy, with practices that differ from country to country. The major chemotherapeutic agents approved for lung cancer include cyclophosphamide, doxorubicin, vincristine, cisplatin and mitomycin-C [60–62]. Almost all of these agents share the common feature that they induce apoptosis in the cell by triggering DNA damage in various ways.

Cyclophosphamide (Cytoxan, Neosar) is an immunosuppressant used for the treatment of lung cancer, and its metabolite, phosphoramide mustard, is the molecular structure form which exerts the actual effect. This agent alters DNA structure by forming irreversible cross-links between N-7 atoms of the guanine base in the DNA strand. This altered DNA structure stimulates intracellular apoptotic pathways, allowing the cell to undergo apoptosis [63].

Doxorubicin, sold under the commercial name Adriamycin, is another chemical agent that can be administered via intravascular route and interacts with DNA by intercalation. This agent prevents the biosynthesis of DNA macromolecules, inhibits DNA replication by stabilising topoisomerases and thereby shows the anti-cancer effect [64, 65].

Cisplatin is a platinum molecule with two chloride ions. This agent interferes with DNA, prevents replication and induces apoptosis in cells that are rapidly proliferating. Because of the different chloride concentration in intracellular and extracellular environment, cisplatin readily enters the cell and interacts with the water molecule to form a complex. This complex replaces the N-heterocyclic bases in DNA and has a particularly strong binding effect on guanine. This new structure forms the cis-[PtCl(NH3)2(N7-ACV)]$^+$ structure. In this situation, DNA repair mechanisms cannot work, and degradation of DNA is initiated in apoptotic cells [66, 67].

Mitomycin-C is another bactericidal chemotherapeutic agent. The mechanism of action of this agent is to generate DNA damage by alkylating the guanine nucleotide in 5′-CpG-3′ sequence via cross-links. Mitomycin-C exerts the anti-cancer effect by activating apoptotic pathways [68].

Another treatment modality employed for the treatment of lung cancers is radiation therapy. Radiotherapy is a radiation-based application that utilises ionised radiation, such as high-energy

X or gamma radiation. Radiotherapy can be applied before or after surgery and can also be combined with chemotherapy, depending on the localisation and stage of the tumours that are being treated. In this therapy, high-energy radiation beams cause DNA damage in the cell and result in apoptosis [69, 70].

The abovementioned therapeutic approaches share a common mechanism of action, in that almost all chemotherapeutic agents and radiotherapies induce DNA damage by activating apoptotic pathways and destroy the tumour with the help of apoptosis [71]. However, this requires the presence of intact apoptotic pathways; in other words, apoptotic pathways should not have been inhibited in order for these treatments to be effective. If anti-apoptotic mechanisms are activated, these treatments often fail, and drug resistance may develop. Specific targets of certain chemotherapeutic agents may be located in base sequences that undergo methylation, and motif changes may occur in the relevant DNA sequence due to methylation. In this case, chemotherapeutic agents may prove to be ineffective.

DNA methylations, chromosome acetylations and inhibited apoptotic pathways are among the reasons of resistance to therapeutic agents and radiotherapy. Studies have shown that epigenetic agents are highly promising in terms of overcoming the resistance to chemotherapy in various tumours [72]. A good understanding of acetylation, methylation and apoptosis mechanisms will allow developing more effective and targeted novel molecules.

10. Potential novel treatment approaches

Excision of the tumour tissue and the surrounding lymph nodes with the most recent surgical approach remains the optimal treatment in lung cancers. However, this may not always be possible owing to the anatomic location, spread pattern and metastasis status of the cancer. Chemotherapy or radiotherapy or both may be used in such cases. Response to treatment, however, is often not promising [73]. It is at this point where epigenetic alterations during cancer development emerge as therapeutic options. Due to their reversible characteristics, epigenetic modifications are therapeutic targets which may prove to have very good anti-cancer effects. For this reason, the US Food and Drug Administration (FDA) and European Medicines Evaluation Agency (EMEA) have started granting approval for certain drugs such as histone deacetylation inhibitors and DNA methyltransferase inhibitors. Among these, inhibitors of DNA methylation are the most effective treatment options and they appear to be effective in lung cancer as well [36].

DNA DNMTs are molecules that transfer methyl groups to cytosines via S-adenosyl methionine (SAM). Hypo- and hyper-methylation of DNA may occur in any cancer cell and silence tumour suppressor genes or inactivate T-cell recognition genes, which provide immune response, or affect the genes that trigger metastasis, angiogenesis and invasion [74]. The most important investigational DNA methyltransferase inhibitors and their analogues are presented in **Table 1** [74].

CI-994 is one of the candidate therapeutics in clinical testing phase. CI-994 is an orally bioavailable histone deacetylase (HDAC) inhibitor that causes histone hyperacetylation in viable cells. CI-994 shows inhibitory effects based on the concentration of HDAC1 and HDAC2.

DNA Methyltransferase Inhibitors Substance Group	Substance Name
Nucleoside analogs	Decitabine (5-aza-2'-deoxycytidine,
	Dacogen®, DAC)[1,2]
	Azacitidine (5-azacitidine, Vidaza®)[1,2]
	5-aza-fluoro-2'-deoxycytidine (FCdR)
	CC486 (oral azacitidine)
	Guadecitabine (SGI-110)
	Sinefungin
	Zebularine
Antisense oligonucleotide	DNMT1 ASO
(ASO) inhibitors	MG98
Others	1-Hydrazinophthalazine
	CBC12
	Epigallocatechin gallate (EGCG)
	Procainamide
	Psammaplin A
	RG-108
	SGI-1027
	Thioguanine

[1]Food and Drug Administration (FDA)-approve drugs, [2]European Medicine Agency (EMA)-approved drugs

Table 1. DNA methyltransferase inhibitors and their analogues [74].

Specifically, it mediates the arrest of cell cycle at G1, inhibits proliferation and induces apoptosis both *in vitro* and *in vivo* [75, 76].

FDA-approved epigenetic therapy agents are shown in **Table 2** together with their indications and year of approval [77].

Acetylation-based drug study is an early phase 2 trial of vorinostat (Zolinza®) [78]. Vorinostat is an HDAC inhibitor from the hydroxamate group. In this phase 2 study, vorinostat was

FDA-approved epigenetic therapy

Agent	Class	Approval Date	Indication
Azacitidine	DNMT Inhibitor	2004	Myelodysplastic syndrome
Decitabine	DNMT Inhibitor	2006	Myelodysplastic syndrome
Vorinostat	Pan-HDAC Inhibitor	2006	Cutaneous T-cell lymphoma
Romidepsin	Class I HDAC Inhibitor	2009	Cutaneous T-cell lymphoma
Bellnostat	Pan-HDAC Inhibitor	2014	Multiple myeloma
Panobinostat	Pan-HDAC Inhibitor	2015	Peripheral T-cell lymphoma

HDAC, Histone Deacetylase: DNMT, DNA methyltransferases.

Table 2. FDA-approved epigenetic therapy agents [77].

evaluated in breast, colorectal, and non-small lung cell cancers; however, adequate clinical response was not obtained, and authors reported that studies are to continue to determine appropriate doses [78]. While having a wide spectrum of tolerable side effects, it is a promising molecule in terms of chemotherapeutic utility [79].

In addition to survivin gene vaccines, a study has been conducted to target the methylated oligonucleotides of the survivin gene in non-small cell lung cancer. The study in question aimed to break down the apoptosis resistance of NSCLC by interfering with the survivin gene expression via oligonucleotides called SurKex1, which are specific to the promoter region of the methylated survivin gene. Data from this study were the first to show the utility of SurKex1 owing to its downregulating effects on survivin expression by means of DNMT1 activation [80, 81]. This study, although conducted in a cell-culture setting, is promising with regard to targeted survivin gene therapy in the near future.

Chemotherapy combined with immunotherapy may also be effective in treatment. High rates of response to treatment were demonstrated through PD-1 blockade via activated IFN signals by hypomethylation in treatments combined with IRF1/7 following treatment with decitabine (5-aza-2′-deoxycytidine or 5-Aza-Cdr or DAC), which was the first cytosine analogue synthesised by Pliml and Sorm in 1960. DAC, known to inhibit DNMT during cell division, is also a candidate for use in cancer therapy as an FDA-approved promoter hypomethylation inhibitor [82, 83].

Cystatin A (CSTA), a member of the type 1 cystatin superfamily, is essential to protect cells from cytoplasmic proteolysis and is mainly expressed in epithelial and lymphoid tissue. Furthermore, while cathepsins B, H and L, CSTA and cytoskeleton are known to be involved as tumour suppressors in oesophageal cancers, they have been found to exert such effects also in lung cancers. Histone methylation and acetylation play an important role in CSTA gene silencing in lung cancers. DAC treatments have an inhibitory effect on DNMT1, which is responsible for replicating DNA in a methylated form during replication. While limited CSTA expression is associated with high grades in squamous cell carcinoma (SCC), silencing in CSTA promoter region has also been demonstrated in the absence of CpG islands through epigenetic mechanisms such as partial methylation [84]. This renders DNMT1s a good target for novel treatment approaches.

In lung cancers, miR-9-3 hypermethylation occurs and the resulting downregulation of miR-9-3 expression leads to poor prognosis. Sulforaphane (SFN), a natural plant-derived molecule with anti-cancer properties, has been reported to decrease miR-9-3 methylation by attenuating DNMT activity in lung cancers and has a potential effect in improving the cancer prognosis [85].

It has been shown that Runx transcription factors (Runx1, Runx2 and Runx3), which play a critical role in organogenesis and cell differentiation pathways, are involved in lung cancers as they cause epigenetic silencing of a tumour growth inhibitor called BMP-3B. In this respect, downregulation of BMP-3B and lung cancers are closely related. Therefore, Runx transcription factors now appear to be a potential epigenetic target in lung cancers [86].

MARVELD1, a recently identified nuclear factor, is known to be extensively expressed in all human tissues and downregulated via promoter methylation in multiple cancer tissues. By working in combination with DNA methylation and histone acetylations, the epigenetic silencing of MARVELD1 leads to a decreased expression, causing unfavourable effects on

histopathology and malignancy in lung cancers. This decreased MARVELD1 status in lung cancers eliminates the NMD complex-forming activity with UPF1/SMG1, resulting in premature termination codons and non-functional RNA. Epigenetic MARVELD1 silencing, which may serve as a diagnostic biomarker in lung cancers, appears to be a good target for anti-tumourigenesis [87].

Highly tumourigenic stem-like cells, which are thought to be tumour-initiating cells, cause the initiation, recurrence and drug resistance of cancers. Ca + 2/calmodulin-dependent protein kinase IIy (CaMKIIy), which is abnormally overexpressed in highly tumourigenic stem-like cells, is also associated with poor prognosis in lung cancers. Oct4 is one of the mRNA expression factors for pluripotent stem cells and regulates the differentiation of these cells by inhibiting CaMKIIy through epigenetic regulation. Therefore, Oct4 may be considered as a novel target approach in lung cancers [88].

The long non-coding RNAs are now also thought to offer a potential biomarker in non-small cell lung cancers. Studies have shown that they are particularly increased in non-small cell lung cancers. Because such RNAs occur mainly through methylation at DNA-gene level, long non-coding RNAs appear to be good markers and targets for novel treatment approaches in non-small cell lung cancers with regard to epigenetic mechanisms [89].

Among lung cancers, the metastatic risk is high in adenocarcinomas. In a study conducted to reveal possible biomarkers and therapeutic agent targets in these carcinomas, DNA methylation profile was downloaded from Gene Expression Omnibus (GEO) database, and DNA methylation profile of lung adenocarcinoma was investigated. This study concluded that methylated PTPRF, HOXD3, HOXD13 and CACNA1A genes may be potential biomarkers for the diagnosis and treatment of lung adenomas [90].

11. Conclusions

In light of all the information described earlier, one may conclude that acetylation at histone level and DNA methylations may be potential biomarkers and also good target molecules for treatment, particularly in lung cancers. Especially, the promoter regions of several tumour suppressor genes, and regions such as exon 1, although to a smaller extent, are inactivated through methylation. While the DNMT1 enzyme is in the position of a general target for inhibition to prevent these methylations, approaches such as inactivation of certain specific genes, oncogenes or apoptosis-inhibiting genes by means of methylated DNA oligo-primers appear to be a considerably good option. In the future, a combination of all these possibilities may allow treatments with significantly reduced side effects compared to current treatments as well as improved targeted approaches which destruct or prevent the progression of tumours.

Conflict of interest

There is no conflict of interest.

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The Immune Regulatory Role of Cytokine-Induced Killer Cells Treatment on Non-Small Cell Lung Cancer Patients

Li Zhang and Yanyan Pan

Additional information is available at the end of the chapter

Abstract

Although a great progress has been made in surgery, radiotherapy and chemotherapy for non-small cell lung cancer (NSCLC), the 5-year overall survival rate (OS) remains unsatisfactory (approximately 15%). Recently, cytokine-induced killer (CIK) cells treatment as an adoptive immunotherapy has great promises in the scenario of potential new approaches for the treatment of lung tumors. Adaptive and innate cellular immunity are all important for inhibiting tumor growth and the clearance of cancer. The abilities to efficiently kill tumor cells and promote immune responses are the ultimate basic ability requested to CIK cells treatment. Therefore, we conducted a systematic review to evaluate the immunoregulation of CIK cells treatment in NSCLC patients to provide an objective reference for clinical decision-making.

Keywords: CIK, immune regulatory, NSCLC

1. Introduction

Lung cancer is a major cause of diagnosed cancer worldwide with a 5-year survival rate of less than 15% [1, 2]. Non-small cell lung cancer (NSCLC) accounts for about 80–85% of lung malignancies and shows high morbidity, high mortality and low survival rates. Most of these NSCLC patients are diagnosed at the late stage leading to a poor prognosis.

So far, the predominant applied cancer treatment methods are still surgery, radiation and chemotherapy; however, the effectiveness of these treatments is not completely satisfactory. These methods often have limited effects and often fail to completely remove minimal residual

cancer cells. Surgery is the predominant treatment method against for NSCLC; however, 60–70% of patients who receive surgery may eventually develop loco-regional recurrence or distant metastasis [3]. Chemotherapy may be useful, but drug resistance and adverse effects still remain; therefore, chemotherapy cannot be used for most of the patients due to the poor tolerance of the majority of later stage patients. Thus, the more effective and safer treatments are urgently needed [4], and tremendous efforts had been done to find new method that can offer better prospects to eradicate tumors.

Recently, adoptive immunotherapy is an evolving treatment approach based on the use of the immune system to treat cancer. Immunotherapy has become a hot area in cancer research and treatment in China. The rapid ex vivo generation of adoptive immune cells is a simpler, cheaper, and more efficient way to generate a potent immunotherapeutic product [5, 6]. Since the 1980s, there are different forms of adoptive immunotherapy used in clinical trials: lymphokine-activated killer cells (LAK), tumor-infiltrating lymphocytes cells (TIL), natural killer (NK), γ δ T-cells and cytokine-induced killer (CIK) cells, tumor-associated antigen (TAA)-specific cytotoxic T-cell (CTL), and other forms of cells have been extensively employed in adoptive immunotherapy [7].

Among them, CIK cells are a heterogeneous population of ex-vivo expanded T lymphocytes with diverse T-cell receptor (TCR) specificities and are endowed with nonmajor histocompatibility complex (MHC)-restricted activities against tumor cells. The antitumor activity is mainly, even if not exclusively, associated with the CD3$^+$CD56$^+$ cells [8]. The antitumor effects of CIK cells have been described against a number of hematologic and solid malignancies both in vitro and vivo. In vitro, CIK cells can kill tumor cells directly [9–11] and improve apoptosis of tumor cells [12], CIK cells also can reverse the drug resistance of A549/DDP [13], and other study found that CIK can alter the cytokine secretion profiles of some immune cells [14]. In vivo, CIK cells also showed significant antitumor activity in animal studies and clinical trials. In the severe combined immunodeficiency (SCID) mouse model, human CIK cells infusion significantly prolongs the survival of SCID mice when compared with control animals or animals infused with LAK cells [15]. In other studies, using the SCID model, CIK cells have in vivo antitumor activity against a number of hematopoietic and solid tumors [16]. Since 1991, CIK cells were reported by Schmidt-Wolf [15], and several clinical trials have been studied. The application of CIK cells as an adoptive immunotherapy is important for the treatment of cancer, since several clinical studies have confirmed the safety of CIK therapy for patients [17, 18]. Numerous clinical studies have been recently performed, whereby adjuvant infusions of CIK cells following surgical resection or chemotherapy demonstrated a significant increase in survival time [19, 20]. The first clinical study included 10 patients with metastatic renal carcinoma, colorectal cancer and lymphoma. One patient with lymphoma obtained a complete response while six patients had progressive diseases and three patients did not experience any change [21, 22]. Other clinical trials subsequently confirmed the safety and benefit of CIK cell-based therapy along with demonstration of initial clinical activity [23, 24]. But, in a recent review, it is reported that patients with localized NSCLC (stages I–III) using immunotherapy (excluding checkpoint inhibitors) did not get a survival benefit [25].

The host immune system plays a critical role in tumor surveillance and rejection. Innate and adaptive cellular immunity are all important for against tumor growth and the clearance of

cancer. Lung cancer is not typically regarded as an "immunogenic" malignancy, a growing body of evidence suggests that immune responses to lung tumors might be present, and their magnitude might correlate with patient outcome. According to previous studies, cancer patients have some dysfunctions in cellular immunity, including innate and adaptive immune responses [26]. Because of the low immune functions of lung cancer patients, effective immune response cannot be achieved. That is one of the reasons that malignant tumors are incurable [27, 28]. Autoimmune disorders in peripheral blood of lung cancer patients are demonstrated by lower levels of CD4+T, NK cells, DCs and higher levels of CD8+T cells [29–31]. Several studies showed that in peripheral blood, increased number of inhibitory cells such as regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and inhibitory molecular such as PD-1 TIM-3 expressed on lymphocytes has been observed in patients with NSCLC, and other cancers [32–34]. Otherwise, in tumors tissue there are local immunological changes. Tumor-infiltrating lymphocytes (TILs) may play an important role in cell-mediated immunological destruction of tumors. Many studies demonstrated that there are more T-cells in tumors tissue in NSCLC compared with that in normal tissue [35, 36]. Accumulating evidence shows that levels of TILs are associated with improved recurrence-free survival in NSCLC patients as well as a reduced likelihood of systemic recurrence [37, 38]. A high density of tumor-infiltrating Tregs was reported to be associated with the recurrence of resected NSCLC [39].

The abilities to efficiently kill tumor cells and promote immune responses are the ultimate basic ability requested to adoptive immunotherapy. The number of immune cells particularly Th1 cells, CD8+ T-cells, and NK, NK T-cells is associated with the survival of cancer patients. Such antitumor cellular immune responses can be greatly enhanced by adoptive transfer of CIK cells [40, 41]. However, the immune regulation role of CIK cells treatment remains controversial. Therefore, in the present study, we conducted a review to evaluate the immunoregulation of CIK cells treatment in NSCLC patients, in order to provide an objective reference for clinical decision-making.

2. Immunoregulation of CIK cells treatment in NSCLC patients

2.1. The changes of lymphocytes of NSCLC patients in peripheral blood

According to previous studies, cancer patients exhibit certain dysfunctions in cellular immunity, including innate and adaptive immune responses [26]. Due to the low immune function displayed by patients with lung cancer, effective immune response cannot be achieved, and this is one of the reasons why malignant tumors are incurable [27, 28]. Autoimmune disorders in patients with lung cancer are demonstrated by reduced levels of CD4+ and CD3+CD56+ cells, and increased levels of CD8+ cells [29, 30]. The response of the human immune system against tumors mainly depends on cellular immunity [42]. CD3+ T-cells are mature T-cells, while CD4+ T-cells are considered to have a predefined role as Th cells [43]. It has been demonstrated that cytotoxicity against tumors is dependent on an appropriate interaction between CD4+ and CD8+ T-cells [44]. However, the ratios of T lymphocyte subsets in peripheral blood are usually disordered in cancer patients [45, 46]. The proportion of these cells in the human body must remain constant in order to maintain its optimal state of balance and participate

in cellular immune surveillance [47]. NK and NK T-cells are effector cells, which are involved in the immune response against tumors during the early stages of tumor development [48]. These cells do not require any specific antibodies or presensitized lymphocytes to exert their function and may be rapidly activated to suppress and destroy a variety of tumor cells [49]. In addition, NK and NK T-cells are more lethal upon being activated by lymphokines [49].

Many studies including our study found that the average percentage of $CD3^+CD4^+$, $CD3^+$ and NK T-cells, as well as the levels of IFN-γ following several treatment courses, were significantly higher than the values observed prior to CIK cells treatment [50–52]. Of course, there are studies found that there are no changes after CIK cells treatment [25]. Here, we have to discuss about the course of CIK cells treatment. Fan et al. found that the median OS of the CIK cells treatment more than four cycles subgroup was significantly longer than that of less than four cycles subgroup [53]. Shi et al. [54] observed that the percentage of $CD3^+$ and $CD4^+$ cells, and the ratio of $CD4^+/CD8^+$ cells were significantly higher following the first course of CIK cells therapy, compared with the values prior to treatment. However, in our study, the percentages of $CD3^+$ and $CD3^+CD4^+$ cells were observed to be no change following only one course of CIK cells therapy, which may be due to the difference in the detection time point (1 month in our study vs. 2 weeks in the study by Shi et al. subsequent to each course), and this result was consistent with others. Jin et al. [55] reported that only one treatment course of CIK cells treatment was unable to improve the immune function in patients with lung cancer. Several researches have reported that $CD3^+$, $CD3^+CD4^+$ and other immune cells peaked at 4 weeks following CIK cells treatment, while circulating CIK cells persisted for ≤2 weeks following infusion [22]. Those studies suggested that several courses of CIK cells treatment would be required to achieve a stable effect [22, 55]. Therefore, to gain therapeutic efficacy, multiple courses of therapy should be administered to the patients.

Otherwise, the treatment done before CIK cells treatment may affect the immune status of NSCLS patients [50]. Thus, the treatments administered to the patients prior to CIK cells therapy seemed to affect the outcome, since more courses were required to achieve effective antitumor immune responses. At present, there is controversy regarding the number of cells required to be infused in CIK cells therapy, since the antitumor activity of CIK cells is mainly associated with the $CD3^+CD56^+$ fraction, rather than the $CD8^+$ fraction, which constitutes the highest percentage of CIK cells [56]. A previous study suggested that the improvements in immune function exerted by CIK cells were affected by the number of CIK cells [22]. In our study, we found that the number of $CD3^+CD4^+$ and $CD3^+$ T lymphocytes did not change following each course of CIK cells treatment. These demonstrated that the number of CIK cells did not affect lymphocyte-associated functions. By contrast, the number of NK and NK T-cells increased with the increase in the percentage of $CD3^+CD56^+$ cells, which enabled the patients to effectively kill tumor cells.

In most studies, patients received autologous CIK cells, allogeneic CIK cells may be applicable in the combination treatment in NSCLC [57, 58], median progression-free survival (mPFS) of allogeneic CIK cells treatment group was significantly longer that of control group [57]. The study found that the levels of IL-2 and IFN-γ in serum did not differ significantly between the two groups both before and after the treatment. Moreover, there were

no obvious changes in the percentage of CD3^{+}, CD3^{+}CD4^{+}, CD3^{+}CD8^{+}, CD4^{+}/CD8^{+} T-cell ratio and NK cells before and after treatment. Wang et al. found that after allogeneic CIK cells treatment, the outcomes of immune function remained unchanged, but the median OS was higher in NSCLC patients receiving allogeneic CIK cells than control group [58]. The CIK cells used in clinical treatment are mostly induced by PBMC in the peripheral blood of patients with tumors. Autologous CIK cells may have the ability to recognize the surface markers of tumor cells and have strong anti-tumor activity. However, in recent years, it has been suggested that low immunity and poor activity of immune cell in patients with tumors may affect the efficacy of CIK cell therapy. Comparative studies of autologous and allogeneic CIK cells treatment have not been reported in lung cancer clinical study. One study had found that semi-allogeneic DC-CIK cells had a stronger anti-tumor effect than did autologous CIK cells in vitro [59]. The reason for this is that the immune function of cancer patients is broken. The CIK cells induced by PBMC in tumor patients are significantly reduced in both quantity and biological activity.

2.2. The changes of Tregs and MDSCs of NSCLC patients in peripheral blood

The response of the human immune system against tumors mainly depends on cellular immunity [42]. However, our immune system does not work effectively in the setting of malignancy. The reason for this may be the existence of immune suppression [60–62].

Tregs have an important role in suppressing adaptive immune responses and maintaining immune tolerance [63]. At the same time, the existence of Tregs may downregulate tumor-specific immunity. Tregs interact with various immune cell types, CD8^{+} T-cells [64], natural killer (NK) cells [65], and natural killer T (NKT) cells [66], thus, Tregs may have an important impact on cancer immune escape. Several studies have reported that the number of Tregs has increased in patients with NSCLC and other cancers [33, 67, 68]. It was reported that the percentage of Tregs was significantly reduced at week 2 after CIK cells treatment compared with the baseline and remained low at week 4 [51]. The decreasing of Treg was related with treatment cycle. Baodan Yu et al. found that significant reduction of Tregs frequency were presented in patients received with more than three cycles of CIK cells treatment compared with patients with less than three cycles of treatment [69]. In addition, inhibitory cytokines TGF-β as well as IL-10 were also decreased [51, 69].

MDSCs are heterogeneous population of myeloid cells known to exhibit potent suppression of T-cell proliferation and cytokine production [70–72]. Several evidence showed that MDSCs have a role in lung tumor growth and progression [73, 74]. In NSCLC patients MDSCs were reported in the peripheral blood [75, 76] and MDSCs were significantly higher compared with the healthy volunteers and were associated with poorer PFS [77]. In case of SCLC, preliminary results from a randomized phase II trial have shown that all-trans-retinoic acid (ATRA)-induced depletion of MDSCs may increase the immune response to DC-based vaccination to 42% [78]. Two Chinese articles found that CIK cells treatment can decrease the level of MDSCs in peripheral blood in malignant melanoma and gastrointestinal carcinomas patients. But, we did not find any literature to study the role of CIK cells on MDSCs in NSCLC patients.

2.3. The changes of immune cells of NSCLC patients in tumor tissue

A malignant tumor is not merely an accumulation of neoplastic cells, but also constitutes a microenvironment including endothelial cells, fibroblasts, structural components, and infiltrating immune cells that impact tumor development, invasion, metastasis, and outcome [79]. More recently, Fridman et al. reviewed that TILs was associated with clinical outcome in several cancers, including lung cancer [80]. All immune cell types might be found in a tumor such as CD4+ T-cells, CD8+ T-cells, macrophages, dendritic cells (DCs), mast cells, NK cells, memory cells, Tregs cells.

CD4+ T and CD8+ cells are the two major subsets of T lymphocytes and have different roles on tumor immunity within the tumor tissue [81]. Wakabayashi et al. [82] found that CD4+ T-cells but not CD8+ T-cells in cancer cell nests are associated with a better prognosis in NSCLC patients. Another study found that infiltrating CD8+ T-cells and CD4+ T-cells in NSCLC may work together to suppress cancer progression [83]. MDSCs were found in lymph nodes and tumor tissue of patients with NSCLC [84]. In mouse model, researchers found that CIK cells can inhibit the accumulation of MDSCs in the tumor [85]. But, we did not find any literature to study the role of CIK cells on infiltrating T-cells and MDSCs in patients with NSCLC. This may have been the result of the difficult access to tumor tissue, and relatively little research was done in vivo about the CIK cells, especially the studies published in high-level journals. Our research team may add these studies to their work in the future.

2.4. Immune checkpoint in NSCLC patients

In the setting of malignancy, immune suppression exists. Except the abovementioned aspects, immune checkpoint is another essential mechanism of immune suppression. These immune checkpoint included cytotoxic T lymphocyte antigen 4 (CTLA4), programmed death protein 1 (PD-1), lymphocyte activation gene 3 protein (LAG3), T-cell immunoglobulin domain and mucin domain 3 (TIM3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and B and T lymphocyte attenuator (BTLA) [86]. They can inhibit the development and proliferation of lymphocytes [87]. These checkpoint expressed on T-cells was found in peripheral blood and tumor tissue have limited ability to effectively eliminate tumors, have gained considerable attention and PD-1 expression on peripheral blood T-cell subsets correlates with prognosis in non-small cell lung cancer [88, 89]. These molecules were found expressed on CIK cells [60]. The majority of these molecules, except BTLA were increased during CIK cells culture. But articles about the effect of CIK cells treatment on these molecules was very little in NSCLC patients, we only found one Chinese article found that after CIK cells treatment, the expression of PD-1 in peripheral blood decreased, and the decreasing was associated with the clinical stage of NSCLC.

2.5. Cytokines in NSCLC patients

In the process of ex-vivo expansion, cytokines play a decisive role in the differentiation and function of CIK. Only the addition of cytokines in vitro can induce peripheral blood monocytes (PBMCs) to differentiate into CIK cells. Importantly, different cultures affected the toxicity of CIK cells [15]. In the process of killing tumors in vivo, CIK can secrete a large number of

IL-2, IFN-γ, TNF-α, and GM-CSF and other cytokines. It can not only play an anti-tumor role directly, but also regulate the immune system of the body, and to stimulate cell proliferation and differentiation, and further enhance the cytotoxic activity of immune cells. Immune balance is controlled by the balance of cytokines produced by two distinct helper T-cell subsets, Th1 and Th2 cells [90, 91]. Our study found that the levels of IFN-γ following several treatment courses were significantly higher than the values observed prior to CIK cells treatment. The levels of IL-2, in vitro and in vivo, were elevated in CIK cells treatment group [12]. The serum levels of IL-4 and IL-6 in tumor-bearing patients were elevated after immunotherapy, and IFN-γ and IL-6 levels in patients with resected NSCLC were significantly increased. Otherwise, Li et al. have also shown that CIK cells treatment increased Th1 cytokines in patients who have no progression, but in patients who had developed metastasis had no change [92]. Successful immunotherapeutic interventions should overcome Th2 immunity by promoting and restoring antitumor Th1 immune response to achieve better clinical benefits [93].

3. Conclusion

In recent years, with the gradual clearer of tumor immunity regulation mechanism and the continuous improvement of gene transformation technology, tumor immunotherapy has been developed unprecedentedly. From the nonspecific immune stimulating agent to tumor vaccine, then monoclonal antibody and adoptive cellular immunotherapy for immune checkpoint blockade, therapeutic immune technology innovation provides more weapons for the human resistance tumor. Especially in the recent 5 years, with the rise of immunotherapy for immunological checkpoints and CAR-T-cell, immunotherapy has become more and more popular. Nonspecific immunotherapy, such as CIK, DC-CIK or NK cells, is widely used in China at present. These kinds of innate immune cells as the first immune defense cells, although the specific killing effect may be less than CAR-T, TCR-T, but these nonspecific cells play a positive role in lowering recurrence rate, improving tolerance of chemotherapy, improving life quality and prolonging the period of survival. However, the curative effect of recurrent and refractory tumors is limited to a small number of typical cases, and there is no evidence-based supports for prospective, randomized controlled trials. Besides, the combination of PD-1 antibody and CTLA-4 antibody is also easy to cause autoimmunity. The instability of tumor cells is also easy to form tumor heterogeneity. There is some progress in the research of CAR-T in the hematologic malignancies, but there are many problems in the treatment of solid tumors for CAR-T. Generally speaking, tumor immunotherapy is progressing faster and has many advantages. But if we want to apply for large-scale clinical application, we need to do a lot of research work.

Several articles had reported the safety and efficacy of CIK-cell therapy. Current studies on the immunomodulatory effects of CIK-cell therapy have focused on T lymphocytes and Treg in peripheral blood. It is much more challenging to study the relationship between CIK cells and immune system. Immune system is dynastic and complex, and a lot of other aspects are in the volume of the complex. The role of CIK cells on immune responses was associated with host factors. Not only cellular immunity we mentioned above but also humoral immunity get involved in tumor immunity. Tumor is characterized by distinct

individual differences, the same disease, different stages, and different pathological types, resulting in different outcomes. Immune system had important roles in these processes. Thus, we evaluate the immune function of the patient and analyze the status of each patient itself, which are significantly related to the final outcome. The ultimate goal of precision immunology for cancer is to select the patients who are most likely to benefit from a particular immunotherapy.

With the development of new technologies to dynamically detect the cancer-immune system interaction and at the same time taking into account the particularity of different groups of people, precision cancer immunology and the evaluation of immune function will be applied more widely, improving diagnosis and treatment of NSCLC patients.

Conflict of interest

No.

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Targeted Photodynamic Therapy for Improved Lung Cancer Treatment

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Additional information is available at the end of the chapter

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Abstract

Cancer develops from the outgrowth of a clonal population of cells with a genetic pathology to evade cell death and exponential proliferation. It has become a global burden with increasing mortality rates. Lung cancer is a major contributor to cancer fatalities. Conventional therapies have shown advances in treating lung cancer, but the successful eradication of cancer lies in targeting both cancer and cancer stem cells. Cancer stem cells (CSCs) are a ration of cells found within the tumour bulk, capable of cancer initiation, therapy resistance, metastasis and cancer relapse. Photodynamic therapy (PDT) has proven effective in treating lung cancer. PDT exerts selective cell death mechanisms toward cancerous cells. With the use of a photosensitizer (PS) which becomes excited upon irradiation with laser light at a specific wavelength, the PS forms reactive oxygen species (ROS) in turn killing neoplastic cells. Leading therapeutic sequel can be obtained by transcending PDT though combination therapies such as immunotherapy and nanotechnology which will enable PDT to target lung CSCs preventing lung cancer recurrence.

Keywords: lung cancer, lung cancer stem cells, PDT, targeted PDT

1. Introduction

Cancer is a global burden affecting millions of people. The yearly death toll for cancer surpasses AIDS, tuberculosis and malaria combined [1]. Cancer is characterised by mutational development of cells that lead to uncontrolled cell proliferation and tumour formation [2]. Tumours are classified according to tissue type and origin [3]. Lung cancer is one of the most frequently diagnosed diseases, having the highest fatality rate amongst all cancers [1]. Carcinoma of the lung arises due to risk factors; such as smoking, corrosive chemical inhalation and air pollution; leading to accumulated mutations of normal lung tissue. These mutations cause

genetic modifications that can alter cell cycle regulation, leading to increased cell proliferation, tissue invasion, tumour formation and metastasis [4]. Lung cancer treatment options are: chemotherapy, radiation, surgery, targeted and immunotherapy [5].

It has been hypothesised that a small group of cells residing within a tumour are responsible for tumour initiation and development. These cells; called cancer stem cells (CSCs); arise from normal stem cells (SCs) that have acquired several mutations, due to their extended life span as compared to differentiated cells [6]. Dysregulation of pathways controlling SCs are seen in CSCs, which lead to exponential cell proliferation, evasion of apoptosis, infinite replication capacity, angiogenesis, metastasis and immune response evasion [7]. CSCs have been identified and characterised using various biochemical assays and techniques. Lung CSCs with tumorigenic potential have been identified [8]. They can be characterised and isolated using CSC identification methods [9, 10]. Due to the identification of CSCs in lung cancer it has become apparent to re-evaluate and develop target specific therapies for lung cancer. Evidence suggests that conventional therapies fail in complete cancer eradication due to lung CSCs and their abilities of drug efflux, treatment resistance and metastasis.

Photodynamic therapy (PDT) is a low cost, minimally invasive therapeutic model that has previously been used for lung cancer treatment. PDT uses a non-toxic photochemical dye/ photosensitizer (PS) that is administered orally or intravenously and absorbed by the cancer. The dye localises in the cellular organelles, whereby upon activation by light at a specific wavelength causes cell death [11, 12]. Even though PDT has shown many successes treating lung cancer [12], there are still some complications that need to be addressed such as photosensitivity and low tumour selectivity [13]. New advancements addressing the complications seen in PDT have been made by developing a PS that is cell specific which can target CSCs in particular by using immunoconjugates and carrier molecules in the form of antibodies (Abs) and nanoparticles (NPs), respectively.

2. Cancer

2.1. Cancer

Malignancy or cancer is a term used for diseased cells. These cells characteristically evade cell death through rapid proliferation and can metastasize by travelling through the blood and lymphatic systems invading distant tissues [14]. Collectively, cancer has more yearly fatalities than diseases such as AIDS, tuberculosis and malaria. According to the International Agency for Research on Cancer the most frequently diagnosed cancers were lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectal (1.4 million, 9.7%). The most prevalent cancer-related fatalities included lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) malignancies. Population growth and ageing affects the cancer related outcome. By 2030, it could be expected that there would be 27 million cases of cancer, 17 million cancer deaths annually and 75 million persons living with cancer within 5 years of diagnosis [1]. Cancer arises from progressive transformation of normal cells that encounter

genomic damages leading to mutations in their DNA sequence. Corruption of the DNA can be endogenous caused by errors in replication of DNA, the intrinsic chemical instability of certain DNA bases or from attack by free radicals generated during metabolism. Exogenous DNA damage can be caused by ionising radiation, UV radiation and chemical carcinogens. Although cells have the ability to repair unwanted changes in the genome, errors may occur leading to permanent mutations. Errors such as inactation of regulatory genes maintaining genomic integrity facilitate additional mutations [15].

Tumorous cells can overpower their normal functioning neighbouring cells eventually forming a tumour as it overcomes normal regulation of cell growth leading to clonal evolution [2]. Neoplastic cells are self-sustainable, making them able to relocate to any space of the body and multiply. This is due to activation of certain enzymes, specifically telomerase, which is normally active only in SCs. Telomeres control cell death by shrinking during every mitoses until the cells eventually die. Therefore cancer cells are able to evade cell death through up regulation of telomerase as it avoids telomere shrinkage, preventing it from shortening leading to elongated telomeres. In addition, telomerase can prevent cell senescence and apoptosis [16].

Cancer classification is based on their tissue type and origin. Carcinomas encompass more than 80% of all cancer cases. These are cells that are epithelial in origin, and usually include breast, colon, prostate and lung. Carcinomas are subdivided into adenocarcinoma and squamous carcinoma [3].

2.2. Lung cancer

Lung carcinomas are neoplastic cells showing unrestrained development of mutated lung cells that are formed in the lung tissue lining the air passages. The mutated cells divide rapidly leading to tumour formation. As tumour formation progress, the numerous abnormal cells start undermining the lungs primary function preventing the lungs from providing the bloodstream with oxygen. Lung cancer can be categorised into two broad groups namely: Small cell lung cancer (SCLC), which is characterised by its neuroendocrine appearance. It encompasses 15% of lung cancer cases. Non-small cell lung cancer (NSCLC), accounts for the remaining 85% of cases. It is classified into subtypes including: adenocarcinoma (38.5%), squamous cell carcinoma (20%), and large cell carcinoma (2.9%). 52% of Patients have a 5 year expectancy when diagnosed with localised disease. Over 52% of patients with distant metastasis at diagnosis have a 5-year survival rate of 3.6% [17].

Regulatory circuits maintaining normal cell proliferation and homeostasis have defects in lung carcinoma. A multistep transformation is followed from a normal lung cell to malignant lung cancer phenotype, altered by a series of genetic and epigenetic modifications, leading to aggressive cancerous expansion. Subsequent to the primary cancer development, constant addition of genetic and epigenetic abnormalities follow during cancer proliferation, leading to tissue invasion, metastasis, and resistance to conventional therapies. Cancer prevention, early detection and treatment rely on the identification and characterisation of these molecular changes. Information on tumour characteristics and genetics will significantly advance prognosis and ideal treatment selection [18].

Contributing carcinogenic risk factors for lung cancer include: smoking, passive smoking and radon; occupational exposures such as asbestos; inhalation of corrosive chemicals like cadmium, silica and vinyl chloride; and long-term and accumulated exposure to air pollution. Lung cancer can also be congenital, where family history of lung cancer increases the risk of development [4].

Therapeutic modalities for NSCLC include: surgery, radiofrequency ablation (RFA), radiation therapy, chemotherapy, targeted therapies and immunotherapy. Therapeutic options depend on the cancer stage, patient's health and lung function and cancer characteristics. Treatments used for SCLC include: chemotherapy, radiation, surgery and palliative care. Surgery is less likely to be a primary treatment for SCLC as by the time diagnoses are made it would have metastasised [5].

3. Cancer stem cells

3.1. The CSC hypothesis

It is hypothesised that tumour development and progression is maintained by a small subset of cancer cells having SC characteristics. These CSCs are capable of self-renewal and differentiation, playing a significant role in malignant proliferation, invasion, metastasis, and tumour recurrence. Cancer cells have accumulated several mutations during their cell cycle, acquiring significant characteristics called the hallmarks of cancer. These specific traits include evasion of growth signalling pathways impeding proliferation, anti-apoptotic functions, infinite replication capacity, angiogenesis and metastasis with distant organ invasion, as well as immune response evasion. In order for a cell to acquire these mutations, its cell cycle needs to be longer than that of somatic cells. Cells that are maintained throughout an organism's lifespan are adult SCs, making them susceptible to neoplastic conversion [19]. SCs divide either symmetrically producing two daughter SCs, or asymmetrically producing one progenitor and one SC, having the ability to differentiate into multiple cell types while self-renewing and overcome senescence [6].

Dysregulation of the pathways maintaining SC function can lead to uncontrolled cell division and differentiation leading to CSC formation and tumour progenitors [7]. A major pathway involved in cell cycle proliferation and arrest is Wnt-β-catenin, which promotes SC renewal by signalling transcription genes. [20]. SC self-renewal is regulated by Notch signalling [21]. The Sonic Hedgehog (Shh) pathway promotes SC proliferation, activating various SCs [7]. Studies have found that these signalling pathways are not always activated in normal SCs but rather in CSCs where the genetic programs governing self-renewal are stimulated in SCs when the need for rejuvenation and repair arises where as in CSCs it is differentially active [22].

3.2. CSC identification and characterisation

Improved identification and isolation of CSCs will lead to enhanced studies on CSCs and targeted therapies. To date, various methods have been implicated in this regard, having different levels of success in common malignancies [23].

First identification of CSCs where made by Bonnet and Dick in 1997, who identified an arrangement of stem like cells that simulated the normal hierarchy of haematopoietic SCs. They identified rare carcinogenic cells in human acute myeloid leukaemia (AML) that was able to repopulate the entire original disease over serial transplantations. The subpopulation characterised by CD34 +ve and CD38 −ve had the capacity to self-renew and differentiate [24]. This study formed the basis for CSC research in both hematologic malignances and solid tumours. Breast CSCs from a solid tumour was first identified by Al-Hajj et al., using CD44 and CD24 markers [25]. Since then, CSCs have been identified in a variety of solid tumours, including lung cancer [26, 27]. Common characteristics from these different tumour types are shared between the isolated CSCs. Characteristics include drug resistance, propagation of tumours, and asymmetric division. CSCs can be isolated and characterised by means of the following methodologies: isolation using CSC-specific cell surface markers by flow cytometry [28]; detection of side-population (SP) phenotypes by Hoechst 33,342 exclusion [29]; assessment of aldehyde dehydrogenase (ALDH) activity [30]; characterisation by tumourigenicity evaluation [31] and stem-ness gene expression and transcriptional factors [32].

3.3. Lung CSCs

Lung cancer's ability to recur, regardless of putative treatment, proposes that a small population of the disease contain the capacity for self-renewal and regeneration. This sub/side population (SP) of CSCs portray tumorigenic potential. With therapeutic targeting, treatments may have the potential to eliminate tumour recurrence [8].

The lung being highly compartmentalised, have led to various epithelial cell types being labelled as presumed lung precursors due to their stem/progenitor cell-like responses to injury. The behaviours and characteristics of these cells also include repopulation of injured tissue. Cell type AEC2 have been characterised as a limited, epithelial progenitor for the alveolus, as they are said to be the progenitor of AEC1, which is involved in gas exchange in the alveolus. Studies have indicated that the bronchio-alveolar stem cell (BASC) a less differentiated cell located in the bronchio-alveolar duct junction act as an injury-responsive, limited progenitor for the distal airway-alveolar epithelium. BASCs have been implicated in lung cancer tumour genesis due to their overexpression of oncogenic K-ras and rapid proliferation by K-ras signalling. Clara cell and AEC2 markers are found in tumour formation of BASCs that have been expressing long term activation of K-ras, both Clara cell secretory protein (CCSP) and surfactant protein C (SP-C) have been identified respectively. Studies have indicated that cancer cells portraying a distinctive combination of the clara cell and AEC2 markers present in BASCs can be isolated from lung tumours. Supporting evidence shows that BASCs constituting of these double positive tumorigenic cells may be responsible for adenocarcinoma development. Along with BASCs and AEC2 being exceedingly receptive to proliferative stimuli, they show resistance to cell damage and injury by expanding within the epithelium following lung tissue damage and repair. These characteristics are critical for both normal tissue and CSCs. As injury resistance of these cells in lung cancer, could serve as a stem cell-like reservoir for generating additional tumours [19].

Lung CSCs can phenotypically be identified and characterised using CSC identification methods. One such method includes the SP phenotyping where efflux of Hoechst 33,342 dye

is measured due to the differential ability of the cancer cells imparted by the ATP-binding cassette family of transporter proteins present on the cellular membrane [9]. Increased ALDH activity is connected to cellular drug resistance, through detoxification of cytotoxic agents and oxidation of retinol to retinoic acid. It is also involved in early SC development and can be used as a reliable CSC marker [8].

Lung CSCs can also be tested for up regulation of SC genes. In a study conducted by Zakaria et al. they investigated CSCs isolated from lung cancer cell lines expressing SC transcription factors Sox2, Oct 3/4, Nanog, c-Myc, and Klf4. Gene expression in the lung CSCs were compared to the expression levels in normal SCs (PHBEC). Sox2, Oct4, c-Myc, and Klf4 were all detected and up-regulated in the CSCs. Currently, specific cell surface markers derived from the surface markers known to be present on normal haematopoietic or embryonic SCs are used to identify and isolate CSCs. For lung CSCs, CD133, CD166, EpCAM, CD90, and CD44 have been used as markers [10].

4. Photodynamic therapy

4.1. Fundamentals of PDT

PDT is a low cost, clinically approved, minimally invasive therapeutic procedure that can exert selective cytotoxic activity toward malignant cells. The procedure involves administration of a photosensitizing agent followed by irradiation at a wavelength corresponding to an absorbance band of the PS. In the presence of oxygen, a series of events lead to direct tumour cell death, damage to the microvasculature and induction of a local inflammatory reaction [11].

Molecular oxygen (O_2) is the terminal electron acceptor of the mitochondrial electron transport chain performing aerobic respiration. In the mitochondrion oxygen serves as an electron acceptor [33]. During PDT a photochemical reaction uses the free O_2, generating a highly reactive product termed singlet oxygen ($^1O_2$) and reactive oxygen species (ROS) which can rapidly cause significant toxicity leading to cell death via apoptosis or necrosis. Ground state/molecular oxygen has two unpaired electrons residing separately in the outermost antibonding orbitals. Depending on the electron configuration there are three possible states for O_2, the ground state of oxygen is called a triplet state $^3O_2$. Singlet oxygen is produced when undergoing photo-oxygenation, by inverting the spin of one of the outermost electrons (**Figure 1**). This type of oxygen is highly reactive and is the predominant cytotoxic agent produced during PDT [34].

A PS or photosensitizing agent is a chemical compound that can be excited by monochromatic light having a specific wavelength matched to an absorption peak of the administered compound. The excited PS subsequently transfers energy to a chosen reactant. This is commonly molecular oxygen [35, 36]. PSs commonly used in cancer are based on the tetra-pyrrole backbone simulating protoporphyrin found in haemoglobin. Naturally occurring tetra-pyrrol structures are found in haem (porphyrins), chlorophyll and bacteriochlorophyll. Synthetically synthesised tetra-pyrroles include phthalocyanines. As pyrrole-ring double bonds are successively reduced starting in porphyrins and going to chlorins and bacteriochlorins, the Q-band
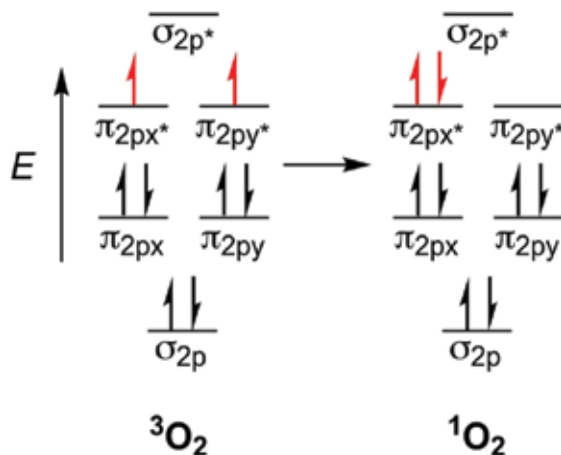
Figure 1. Molecular orbital diagrams showing the electron distribution in triplet and singlet oxygen [34].
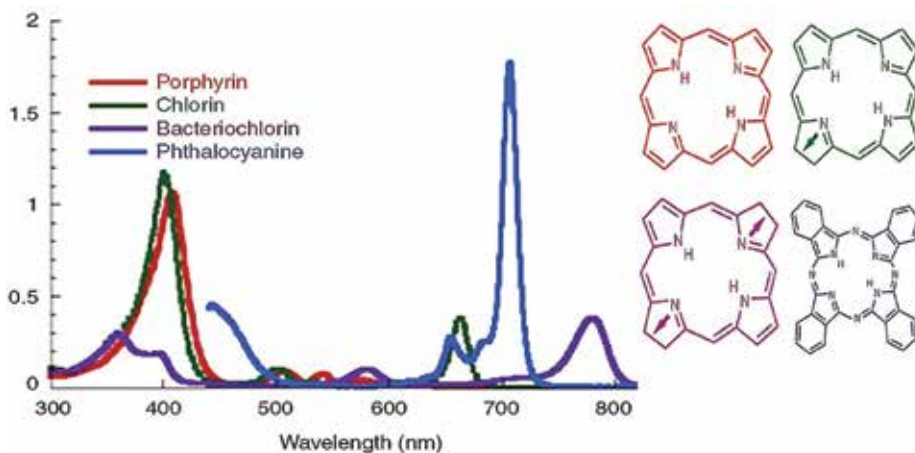

Figure 2. Tetrapyrrole absorption spectra showing porphyrins, chlorins, bacteriochlorins, and phthalocyanines [37].

moves to longer wavelengths and increases in size as seen in **Figure 2** [37]. Indicating that the structure of the PS has an influence on absorbance bands.

Efficient PSs should have a strong absorbance peak ranging from 600 to 800 nm in the deep-red to near-infrared (NIR) spectral region, which will allow for tissue penetration, as penetration tend to increase with wavelength. However, wavelengths longer than NIR are avoided, due to having a lower frequency and delivering too little energy for sufficient oxygen excitation. Ideally a PS should have suitable photo-physical characteristics. It should have a high-quantum yield of triplet formation ($\Phi T \geq 0.5$), a high singlet oxygen quantum yield ($\Phi \Delta \geq 0.5$), a relatively long triplet state lifetime (τT, μs range), and a high triplet-state energy (≥ 94 kJ mol^{-1}). Low dark toxicity and negligible cytotoxicity in the absence of light. Preferential accumulation in diseased/target tissue over healthy tissue. Rapid clearance from the body

post-procedure, to decrease side effects. High chemical stability: single, well-characterised compounds, with a known and constant composition. Soluble in biological media as effective PSs tend to be relatively hydrophobic compounds that rapidly diffuse into tumour cells and localise in intracellular membrane structures such as mitochondria and endoplasmic reticulum (ER). It should produce a marked inflammatory response via apoptosis, causing an immunogenic effect against cancerous cells [36, 38].

Light source and light delivery are fundamental aspects in PDT. The light source depends on tumour location and the PS used. To date visible light ranging from 400 to 900 nm has been used in PDT. It has been noted that longer wavelengths in the visible red spectrum ranging between 600 and 810 nm are preferred due to optimum tissue penetration as well as PS structure mediating the use of red-shifted light. Historically PDT depended on low intensity lasers for a light source due to their valuable characteristics of monochromaticity, coherence, directionality and low power output (<100 mW), which removes the variable of heat that might have an influence on PDT. Lasers emit narrow beams of intense electromagnetic radiation that is monochromatic giving access to the wavelength region for excitation of PSs [37]. Coherence and directionality is correlated to the laser beams' divergence property. This is a qualitative measure of the laser irradiation to remain concentrated over a distance. Another important factor in choosing the light source is the fact that tissues have various optical properties depending on their bio-components. Tissue can both absorb and scatter visible light, this tend to decrease as the wavelength used increases [39].

4.2. Mechanisms of PDT

Three fundamental components act simultaneously in PDT (**Figure 3**): molecular oxygen, a light source and a PS. None of these is individually toxic.

During PDT, when a PS is absorbed it is still in its ground singlet state. A PS reaches its first excited singlet state through wavelength specific light activation. This first excited singlet state is unstable and can either deteriorate through energy loss by emitting fluorescence or, it can reach its excited triplet state accomplished through intersystem crossing of molecular oxygen, which is long lived and more stable [40] (**Figure 4**). In solution, intersystem crossing is increased by the probability of the presence of paramagnetic species such as molecular oxygen. When the PS reaches its excited triplet state it can follow two pathways. These pathways are named Type I and Type II reactions.

Phostosensitizer   Visible red Light   O2

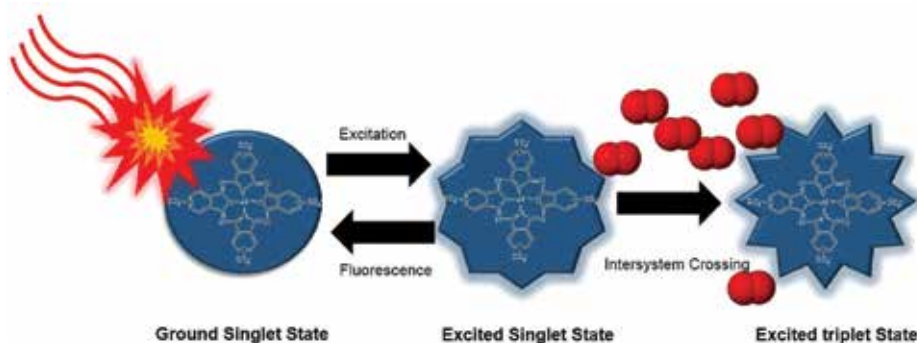**Figure 3.** Fundamental components of PDT.

Figure 4. Activation of a PS in its ground singlet state to its excited triplet state via light activation and intersystem crossing with molecular oxygen.

Type I reactions generate ROS, whereby adjacent biomolecules (i.e. lipids, proteins, and nucleic acids) and the PS in its excited triplet state undergoes an acid-base reaction transferring hydrogen ions. Depending on the target molecule, i.e. lipids, proteins, or nucleic acids, free radicals and radical ions are generated that then react with oxygen forming ROS [41] (**Figure 5**).

Type II reactions are based on a phenomenon called triplet–triplet annihilation. This involves the production of highly reactive singlet oxygen which is also extremely cytotoxic. Singlet oxygen is generated through the PS in its excited triplet state reacting with ground state molecular oxygen [41] (**Figure 6**).

Type I and II reactions happen simultaneously. The oxygen species generated between the two reactions depend on the components, i.e., the PS and amount of oxygen available to react with as well as PS localization in the biomolecules. Type II is considered the primary mechanism of cell death due to singlet oxygen generation. ROS and singlet oxygen have a high reactivity and short half-life, affecting only the biomolecules the PS had localised in or are close to the region where these species are generated, usually within a 20 nm radius. PS localization promotes selective sensitization and is therefore a primary factor in drug release studies to target tissues [41].
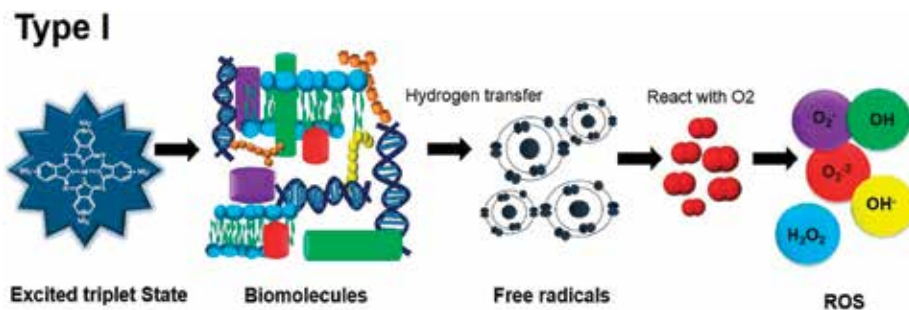

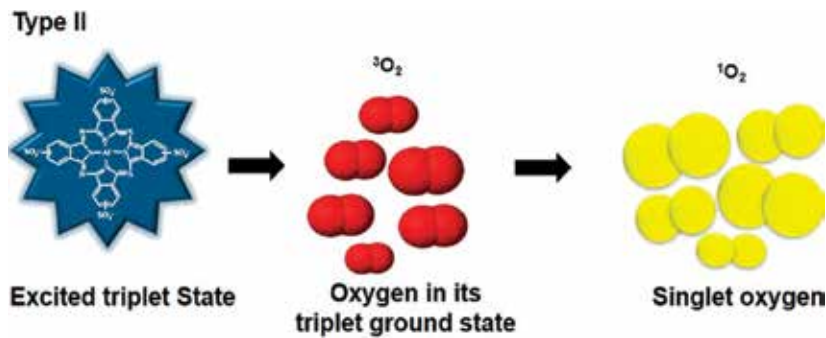**Figure 5.** Type I reaction in PDT.

Figure 6. Type II reaction in PDT.

4.3. PDT and lung cancer

Currently PDT, alone or as an adjunct therapy is increasingly being used to treat thoracic malignancies. Effects exerted include both apoptosis and necrosis, damage to tumour vasculature as well as inducing an inflammatory reaction. It does not lose efficacy with repeated treatments and allow for combination treatment [42].

Porfimer sodium is the PS most commonly used to treat lung and other thoracic malignancies. It has been approved by the US Food and Drug Administration (FDA) for cases of NSCLC where standard therapies are not appropriate and to palliate symptoms from airway obstruction [43]. It is also reported to be a safe and effective neoadjuvant treatment, where it has been reported to significantly decrease tumour size, convert tumour operability and improve complete surgical resection [12]. Over the past decade, prospective clinical studies evaluated a variety of PSs, in treating early and advanced stage NSCLC. Early-stage disease had a complete response range from 72% to an impressive 94 and 100%. Advanced disease, local control and partial response ranged from 78 to 100%, respectively [12]. Key indications for the use of PDT to treat lung cancer include: Intraoperative PDT by transthoracic or thoracoscopic irradiation after tumour resection and complete removal of the macroscopic disease, where the margins in the surgical bed are illuminated before wound closure to treat undetected viable cancer cells, which could lead to a reduction in local recurrence [44]. Interstitial PDT, where intra-tumor light delivery is required to activate the PS, using image guidance and treatment planning, when the tumour is deep-seated and larger than 1 cm [45]. Definitive PDT treatment where indication includes early stage, superficial, and centrally located endobronchial NSCLC tumours, where the treatment option used is admittance of the PS and activation through bronchoscopic irradiation [12].

PDT has shown to be a safe and minimally invasive therapy designed as an anti-cancer drug, but still have room for improvement. Current PSs lack sufficient tumour selectivity which may result in uptake of the PS in non-cancerous tissue that can lead to adverse effects [46]. Another major trial in medicine today is drug delivery, this includes PDT. When administering a PS it is taken up by the blood and lymphatic systems, which can lead to photosensitivity [13].

5. Targeted PDT

5.1. Immunotherapy and PDT

Photoimmunotherapy (PIT) actively and specifically targets antigens via monoclonal antibodies (MAb) or antibody fragments (AbFs) used for drug delivery. PIT uses a PS conjugated to an Ab against a tumour, tumour associated (e.g. CSC) or tumour vasculature antigen [47]. Some of the advantages seen in PIT, compared to conventional cancer treatment such as chemotherapy/chemo-immunotherapy, is that during laser activation of the Ab-PS conjugate there is cell specific cytotoxicity of cancerous cells sparing the surrounding healthy tissue. Additionally PIT is neither immunosuppressive nor does it have an affinity for rapidly dividing cells, making it less likely to develop treatment induced resistance due to neoplastic cells up regulating alternative or circumventive pathways commonly seen in chemotherapy [48].

PIT has shown to be effective in various studies conducted on cancer using a variety of MAbs. This includes a study conducted by Savellano et al., where they conjugated a clinically approved benzoporphyrin derivative (verteporfin) to the anti-EGFR MAb cetuximab. Results showed that conjugated verteporfin had an affinity for EGFR-overexpressing A431 epidermal carcinoma and ovarian cancer cells, killing them via PDT mediated mechanisms, whereas free verteporfin exhibited no specificity [49]. In another PIT study they explored the effectiveness of PIT on metastatic lung carcinoma *in vitro* and *in vivo* using a mouse model. IRDye700DX is a silica-phthalocyanine dye that is extremely hydrophilic. It has an excitation wavelength of 690 nm which is NIR, allowing for enhanced tumour penetration of light. IR700 conjugated to MAbs showed *in vitro* results of target cell specificity with little to no toxic effects on non-target adjacent tissue. Targeted cells demonstrated cell membrane rupture within minutes of exposure to NIR-light activating the Ab-PS conjugate [50, 51]. *In vivo* experiments using trastuzumab-IR700 was used to treat early-stage lung metastases in a murine model. Results indicated specific binding, rapid induction of necrotic cell death, target specific cell death and prevented metastasis by target-specificity [52].

5.2. Nanomedicine and PDT

NPs are biomolecules synthesised for drug delivery and is used in nanomedicine today. Incorporating nanostructured drug delivery systems of PSs conjugated to NPs may have advantages that include improvement of transcytosis across epithelial and endothelial barriers, optimise delivery of low water soluble PSs and co-delivery of PSs into cells [41]. Other advantages of using PS-NP conjugates are defence against enzymatic degradation, controlled PS release into cancer cells, its small size allow for cellular penetration, NPs are biocompatible and photos table [53]. NPs can be classified according to their composition, morphology or structure [54]. Covalently binding the PS to the NP can enhance delivery of the PS to cancerous cells, as well as increase singlet oxygen production [37].

NPs are susceptible to engulfing by macrophages after intravenous administration. This can be overcome by polyethylene glycol (PEG) coating, enhancing bloodstream circulation time and

allowing for tumour accumulation. Studies have showed encouraging results for the use of PS-NP conjugates, whereby different compositions of NPs have been proposed [55]. One such study indicated that the use of dendrimer phthalocyanine (DPc)-encapsulated non active polymeric micelles have been successfully used both *in vivo* and *in vitro* treating human and subcutaneous mouse lung adenocarcinoma A549. Both experimental systems had a significant increase in PS-NP conjugate PDT efficiency as compared to PDT alone, where mitochondrial localization was observed [56]. Another method of enhancing PDT is by improving on the conjugation methods for PSs and NPs. Instead of using PS-NP encapsulation, PS-NP conjugation can be achieved through covalent binding [57]. One NP in particular that can be applied by covalent bonding of PSs to its surface is gold-NPs (Au-NPs). Au-NPs has enhanced surface-plasmon resonance (SPR) effects due to the non-linear optical fields found in metal NPs being very close [58]. Au-NPs have good biocompatibility, versatile surfaces, and unique optical properties [59], whereby their optical field can be enhanced by the SPR by changing the shape of the NP specifically to a ring [60]. Studies have conjugated Au-NPs to Abs, for specific cell surface receptor targeting, in anti-cancer treatments whereby the use of NIR-light produced photo-thermal heat destruction. Results showed a significant increase in apoptosis induction as compared to unconjugated NPs [61]. A drawback in using Ab-NP conjugates alone for cancer treatment was that to induce photo-thermal heat destruction a high power density laser had to be used. This led to unselective damage of normal tissue in the laser path surrounding the target of interest [62]. However, cancer cell death induction using TPDT requires low power lasers that are efficient in activating the PS avoiding destruction of normal tissue. Coating the NP with polyethylene glycol (PEG) have also enhanced conventional methods of Ab conjugation to NPs that led to poor orientation of the functional group of the Ab. NP PEGylation allows for covalent attachment of an Ab to the outer end of the PEG chain, thus maintaining availability of Ab binding sites to cell surface receptors. Studies using this method of Ab conjugation showed efficient internalisation into cancerous cells [63, 64]. TPDT involves
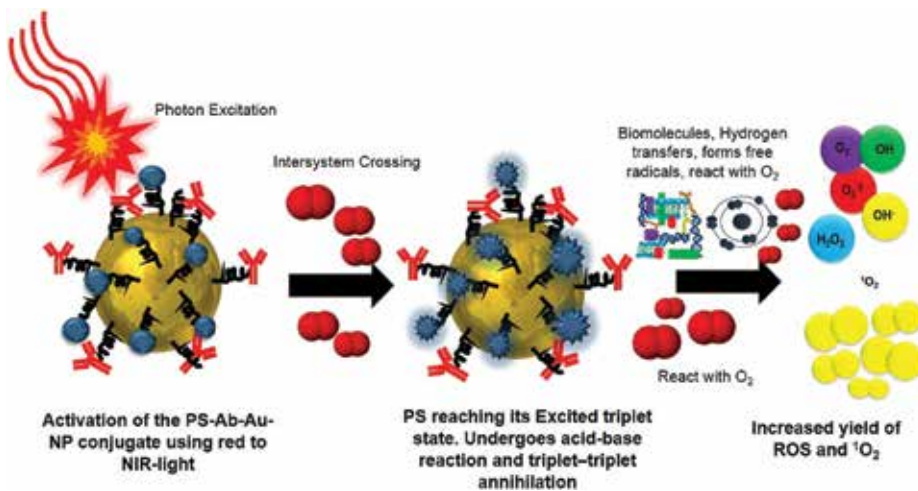

Figure 7. TPDT using a PS-Ab-Au-NP (PEG) conjugate.

the use of either/both an Ab or NP conjugated to a PS. The mechanisms still follow Type I and Type II reactions (**Figure 7**).

6. Conclusion

Despite intensive research and development of therapies, lung cancer still remains a primary contributor to cancer related deaths, with survival rates of patients diagnosed being dismal. Prompt diagnosis and effective treatment will radically improve patient outcome. Due to late diagnoses of lung cancer, conventional therapies show to be ineffective [18]. Lung cancer initiation and progression mechanisms have been identified that will be able to drive future research on molecular and biological targets. Conventional therapies are limited by drug resistance. Characterising and evaluating the mechanisms as well as lung CSCs leading to the acquisition of drug evasion, can aid in the development of therapies that will combat therapeutic resistance [65].

According to the CSC hypothesis, these cells are involved in tumorigenesis. This is because of their stem-cell like abilities that include indefinite self-renewal, slow replication, intrinsic resistance to chemotherapy and radiotherapy, and an ability to give rise to differentiated progeny. Studies have been able to identify CSCs in various cancers, including lung. Lung CSCs have been phenotypically identified using bio-markers typically expressed by normal SCs. Some of the markers include CD133, CD166, CD44 and ALDH1. Molecular pathways regulating SC proliferation, differentiation, and apoptosis are found to be active in CSCs as well, all giving rise to CSCs unique capability of drug evasion and metastasis or cancer relapse [66]. Due to conventional therapeutic strategies only targeting rapidly dividing cells destroying the bulk of the tumour, complete eradication of rare CSCs also need to be addressed. Therapies that aim to identify CSCs and overcome drug resistance due to CSCs having increased levels of efflux pumps need to be developed [27].

A potential therapy that can be advanced to treat CSCs is PDT. PDT involves the use of a nontoxic PS that localises in cellular organelles and when activated using light of a specific wavelength, reacts with oxygen to form free radicals leading to cell death. PSs have an affinity for malignant cells, inducing apoptosis via caspase reactions, mitochondrial damage and cytochrome c release. Unlike chemo and radiation inducing cell death via DNA damage. Another advantage of PDT is that cells that become resistant to chemo and radiation does not cause cross-resistance to PDT and there is no toxic accumulation [67]. Several modes of clinical PDT application has been defined, pertaining to localization and tumour density, as these factors play a role in PDT efficacy. One mode of concern is interstitial PDT, which is used on tumours larger than 1 cm in size [45]. This mode of PDT which is indicated for multicellular tumours has been explored previously *in vitro*. The efficacy of PDT concerning a monolayer as compared to multicellular spheroids indicated that spheroids are more resistant to PDT, however this can be overcome using a dose dependent manner of inducing cell death [68]. Although PDT has successfully been used to treat lung cancer a major pitfall still include low tumour selectivity, especially in a scenario where the lung cancer's genomics are predisposed to malignant metastatic tumours [42].

PIT uses a PS conjugated to and Ab allowing for cell specific cytotoxicity and does not develop treatment induced resistance. Interest has grown in the biomedical field for the use of NPs as a drug delivery vehicle specifically Au-NPs, due to their biocompatibility, high surface area and functionalized facile surface supporting self-assembly of thiolates [69]. Au-NPs can be synthesised to a structure supporting absorption in the far red to NIR wavelength. The combination of using an Ab-NP conjugate allows for all the significant contributions and advantages to be applied in one treatment, TPDT, having improved cell specific targeting as well as allowing significant accumulation of PS in the tumour site by using Abs to direct the PS to CSC specific markers for example, and NPs enhancing PS uptake that can increase singlet oxygen yield and effective cancer/ CSC death. Results indicate that TPDT might prove to be a promising treatment modality for lung cancer and targeting lung CSCs. As TPDT can be used as a primary or adjuvant therapy for lung cancer depending on the morphological state and tumour localization. Targeted PDT can lead to complete cancer eradication and prevent cancer relapse by destroying the bulk of the tumour as well as targeting the underlying CSCs. Improving the overall survival rate of patients diagnosed with lung cancer as well as increase quality of life through minimal side effects when receiving treatment.

Acknowledgements

Conflict of interest

The material in this paper, submitted to *InTechOpen* is original work from the author and is not being considered elsewhere for publication. The authors indicate no potential conflict of interest.

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Chemoresistance of Lung Cancer Cells: 2D and 3D *In Vitro* Models for Anticancer Drug Screening

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Additional information is available at the end of the chapter

Abstract

Chemoresistance of lung cancer cells is a key factor that limits the treatment of lung cancer patients. Patients may initially respond to standard chemotherapy, but this is often followed by rapid development of drug resistance and disease progression. Tumor heterogeneity and the presence of putative cancer stem-like cells (CS-LCs) provide a viable explanation for the chemoresistance of several types of tumors. In this book chapter, we will first describe the current knowledge of the role of both tumor heterogeneity and CS-LCs in lung cancer chemoresistance, tumor progression and metastasis. Next, we will discuss ongoing strategies at the *in vitro* level to screen for more effective anticancer drugs. We will specifically focus in three-dimensional (3D) culture systems (Spheroids and tumorspheres) and their application in anticancer drug discovery for lung cancer.

Keywords: chemoresistance, tumor heterogeneity, cancer stem cells, spheroids tumorspheres, 3D systems

1. Introduction

Lung cancer is the leading cause of cancer death among men and the second leading cause of cancer death among women worldwide [1]. Despite important advances in our knowledge of cancer cell biology and anti-cancer therapies such as chemotherapy, radiotherapy and targeted therapies, the five-year survival rates remain poor (<15%). Lung tumors are broadly classified into small cell lung cancer (SCLC) and non-small lung cancer (NSCLC). SCLCs are defined by neuroendocrine differentiation and small cell morphology of the tumor cells and account for

15–20% of newly diagnosed lung malignancies. Interestingly, SCLC tumors tend to recur as chemoresistant variants and occasionally show progression to a NSCLC phenotype [2]. Regardless of the type, chemoresistance appears in most lung tumors, presenting a challenge to the development of new therapeutic regimes. The failure of the management of lung cancer is largely attributed to the inherent and/or acquired resistance that limits the efficacy of current therapies. Several characteristics of lung tumors have been identified for long time as key driving factors that lead to increased chemoresistance. Among them, mutations, amplifications and overexpression of multidrug-resistant proteins have been investigated *in vitro* using cell lines growing as adherent monolayers (2D systems). Intratumoral heterogeneity was also recognized long time ago as a key factor contributing to chemoresistance and soon tumor spheroids were developed with the aim to replicate *in vitro* "mini-tumor" with more complex and heterogeneous 3D architecture mimicking primary tumors. These tumor spheroids were routinely obtained by culturing cancer cells in serum-containing media under anchorage-independent conditions. The isolation of putative cancer stem cells from solid tumors was done by culturing cancer cells under anchorage-independent condition but in serum-free media (initially with few supplements). Under these conditions, cancer cells grow as "floating tumorspheres" and form complex 3D structures similar to spheroids. It is widely accepted that "floating tumorspheres" are enriched with cancer stem-like cells that are inherently chemoresistant. For clarity and consistency, we will call "spheroids" and "tumorspheres" to masses of cancer cells growing as floating spheres in serum-containing and serum-free media, respectively. The aim of this chapter is to (1) briefly describe the main factors—relevant to 3D *in vitro* models contributing to chemoresistance (intratumoral heterogeneity and the presence of CS-LCs) and (2) discuss the application of spheroids and tumorspheres as tools for screening anticancer drugs targeting chemoresistant cancer cells.

1.1. Intratumoral heterogeneity

Intratumoral heterogeneity is a term that refers to the presence of cells within a tumor with varying degrees of morphology, proliferation rate, ability to metastasize, sensitivity to drugs, dependence on growth signals and tumor initiation/repopulation capacity. It has long been recognized as a salient feature of most cancers and largely associated with tumor relapse. Both genotypic and phenotypic diversity exist within tumors that arise driven by genetic mutations, epigenetic alterations or microenvironmental influence. As a consequence, expansion of selected clones as well as establishment of differentiation hierarchies of cancer stem cells (CSCs) and non-CSCs creates a wide diversity of cells [3]. The basis for the genotypic heterogeneity is the inherent genetic instability of cancer cells and the clonal evolution theory that proposes that a tumor of monoclonal origin may become heterogeneous due to advantageous tumorigenic growth of clonal subpopulations. Over time, as the tumor progresses, cancer cells accumulate different mutations and different clones may compete or evolve in parallel generating a tumor composed of cells with varied genetic imprints. The latter is called branched evolution, and this process has been confirmed by genetic analysis in a variety of tumors [3]. Non-genetic heterogeneity (phenotypic heterogeneity) is the result of microenvironmental pressures due to, for instance, alterations in oxygen, pH and nutrient availability based on their regional location within the primary tumor largely influenced by their relative distance to

blood vessels. Both genotypic and phenotypic heterogeneity are associated with chemoresistance that have a profound impact on the clinical outcome of lung cancer patients. For instance, at the clinical level, it has been recently suggested that increased metabolic heterogeneity should be considered as a high-risk subpopulation for early EGFR TKI failure [4].

1.2. Presence of cancer stem-like cells in lung tumors

The cancer stem cell hypothesis (CSCH) suggests that most cancers contain a rare subpopulation of cancer cells with properties such as indefinite self-renewal, slow replication and ability to give rise to differentiated progeny. These cells possess intrinsic resistance to chemotherapy and radiotherapy and are thought to be responsible for tumor initiation and growth and tumor relapse [5, 6]. The CSCH is a hierarchical model in which cancer stem cells (CSCs) can differentiate into non-cancer stem cells (non-CSCs) but not the other way around. According to this model, eliminating the CSC subpopulation would eventually lead to a cure. This concept has been recently challenged by several alternative models of cancer stem cell biology [7, 8] since experimental evidence demonstrated that cancer cells are extremely plastic [9, 10] and evidence of interconversion between CSCs and non-CSCs was found in a variety of cancer types including lung [11], breast [12] and colon [13] cancers. Contrary to the CSCH, all the alternative models propose that to cure cancer all cancer cells should be eliminated at once. At present, it is safe to assume that tumors may consist of a heterogeneous population of cancer cells with different "stemness" properties ranging from a pure non-CSC phenotype (typically sensitive to conventional anticancer drugs) to a pure CSC phenotype (usually highly resistant to conventional anticancer drugs). *In vitro*, CS-LCs are able to grow in the absence of serum as 3D spheres under anchorage-independent conditions as floating "tumorspheres" (FTs) and it is thought that the ability to form clonal spheres is a unique characteristic of CSCs [14, 15]. Because of its 3D architecture, and the notion that FTs consist of mostly CSCs with inherent chemoresistant properties, they have widely adopted as system models for drug screening (see Section 2.2.2.).

2. *In vitro* models for anticancer drug screening

Several *in vitro* models of lung cancer have been widely used for testing new anticancer drugs. Historically, 2D cultures were introduced first and chemoresistant cells lines were isolated and widely used to screen for more effective anticancer drugs. However, since 2D cultures typically consist of a more homogenous population of cells, 2D systems may not be able to account for intratumoral heterogeneity. As a result that 3D systems would recapitulate more faithfully, the heterogeneous nature of cancer cells existing *in vivo* were soon developed. Before describing these 3D systems, we will briefly discuss 2D systems.

2.1. 2D systems

Some cell lines were found to be inherently resistant to one or more drugs. In addition, several drug-resistant cancer cell lines have been generated in the laboratory to investigate either the underlying mechanism of resistance to a particular drug and/or to screen for alternative drug

able to circumvent the acquired resistant. The next three sections will focus in the development and characterization of cancer-resistant cell lines and their application to identify mechanism of resistance and identification of new targets.

2.1.1. Development of drug-resistant cellular models of lung cancer

Developing models of chemoresistant cancer cells is a well-utilized approach to investigate mechanisms of acquired drug resistance, anticancer drug screening and developing novel drugs to resensitize resistant cancers to apoptotic stimuli. Developing a stable clinically relevant drug-resistant model involves using doses and exposure times consistent with the clinical setting [16]. In order to mimic clinical conditions, developing *in vitro* models of cancer resistance involves chronic exposure to lower concentration of drug with a pulsed treatment strategy that involves cycles of exposure to drug followed by recovery in drug-free medium [17]. Alternatively, continuous selection strategy with increasing doses or acute exposure to high concentration of gefitinib and erlotinib has been used to develop resistant models of NSCLC [18–20].

2.1.2. Characterization of drug-resistant models

Several assays have been used to characterize chemoresistance by comparing the resistant phenotype with the parental cell line. Measurement of drug sensitivity using viability assays such as the MTT assay, flow cytometry analysis to determine cell cycle arrest, clonogenic survival assay, characterization of stemness markers, determination of ALDH activity, expression of EMT markers, characterization of MDR modulators and quantification of cellular uptake of drug used to induce drug resistance are commonly used assays to characterize drug resistance. A dose and time-response curve of cell viability is used to calculate IC_{50}. The fold resistance is calculated by comparing the ratios of IC_{50} of the resistant cells with parental cells [17]. A marked feature of drug-resistant cell lines is enrichment of cancer stem cells, a subpopulation of tumor cells with capacity for self-renewal and tumorigenicity potential. Aldehyde dehydrogenase (ALDH) is involved in differentiation of cancer stem cells and promoting resistance and survival mechanisms [21]. Aberration in regular cell cycle can circumvent or potentiate apoptosis, and drug-resistant cells undergo cell cycle arrest to prevent apoptotic cell death [22]. Epithelial mesenchymal transition has been associated with resistance to gefitinib and erlotinib in NSCLC [23, 24]. Quantifying decreased cellular drug accumulation in the resistant phenotype using inductively coupled plasma mass spectrometry (ICP-MS) due to overexpression of transporter proteins is another novel method of characterizing acquired drug resistance [25, 26].

2.1.3. Identifying mechanisms of resistance and novel drug targets for resistant lung cancer

Epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase (RTK), involved in cellular proliferation is overexpressed in NSCLC and SCLC. Targeting EGFR signaling using tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib was a reasonable clinical success, particularly in patients with EGFR mutations [27, 28]. Acquisition of resistance due to a secondary mutation of EGFR (T790M) is a major therapeutic problem necessitating discovery of novel drugs that can inhibit TKI-resistant NSCLC after developing T790 M mutation [29]. A panel

of 12 NSCLC cell lines comprising wild-type EGFR (TKI-resistant), EGFR mutation with an additional TKI-resistance inducing mutation and EGFR mutation yet sensitive to TKI-inhibitor was used to screen 10 anticancer compounds. All 12 NSCLC cell lines showed inhibition of proliferation with 17-DMAG, an Hsp90 inhibitor and belinostat, a histone-deacetylase inhibitor (HDACi). 17-DMAG and belinostat inhibited EGFR and p-Akt expression in one cell line of each group. Combination of 17-DMAG and belinostat showed synergistic antiproliferative activity and inhibited the growth of TKI-resistant cell lines. These drugs were effective in mice xenografts and completely suppressed tumor growth with the combination being more effective than either drug alone. Immunoblotting of mice tumors showed decreased expression of p-EGFR, total EGFR and p-Akt substantiating a need to validate a combination therapy of 17-DMAG and belinostat in patients with EGFR-TKI-resistant NSCLC [30].

Some mechanisms shown to confer resistance to gefitinib and erlotinib in EGFR-mutated patients in addition to T790 M mutation, stemness and EMT have been shown to be MET amplification, FGFR1 overexpression and IGF1R overexpression [30–32]. These multiple resistance mechanisms can exist in parallel giving rise to heterogeneous resistant cell population. NSCLC grown resistant to erlotinib identified subclones that underwent MET amplification or induced EMT phenotype. MET subclones, while maintaining erlotinib resistance, showed increased sensitivity to MET inhibitors, crizotinib and capmatinib. EMT subclones overexpressed FGFR1 and showed increased sensitivity to FGFR1 inhibitor AZD4547. Inhibitors of MET and FGFR1 showed reduced sensitivity to mixed NSCLC cell line as compared to the individual subclones. This study highlights the coexistence of parallel resistance mechanisms giving rise to heterogeneous resistant population [33]. Anaplastic lymphoma kinase (ALK) gene rearrangements act as oncogenic drivers and are present in a small subset of NSCLC, which are responsive to ALK kinase inhibitors such as ceritinib (LDK378) [34]. Ceritinib-resistant cells were grown by chronic exposure to the drug and assayed for resistance using cell proliferation and viability assays. Ceritinib treatment upregulated Src, an oncogene involved in tumorigenesis and metastatic progression. Knockdown of Src with siRNA in resistant phenotype resensitized cells to ALK inhibition with ceritinib. Resistant ALK-positive cell lines showed sensitivity to Src inhibitor ADZ0530, which has promising therapeutic potential to be explored in patients with ALK-TKI-resistant tumors [35]. In another study, combinatorial treatment of ALK and IGF1R inhibitor was used to overcome crizotinib resistance in ALK-positive lung cancer, positing IGF1R to be an independent drug target in this subset of lung cancer [36]. Interleukin-8 is upregulated in gefitinib-resistant cells and is associated with shorter progression-free survival in EGFR-TKI-treated lung cancer patients. IL-8 expressing EGFR-mutant cell line showed increased phosphorylation of Akt and NF-κB translocation and decreased sensitivity to gefitinib-induced apoptosis. Knocking down IL-8 increased the apoptotic sensitivity to gefitinib. IL-8 expressing cells showed stemness as characterized by ALDH activity, increased expression of Nanog, Oct4 and Sox2 and forming more and larger colonies than controls. This acquisition of stemness was IL-8 dependent, as knocking down IL-8 reversed the stem cell-like properties in EGFR-TKI- resistant cells [37]. Gefitinib-resistant cells were developed to investigate the induction of EMT phenotype associated with EGFR-TKI resistance in NSCLC. While these resistant phenotypes were negative for T790 M mutation or MET-amplification, they had acquired the EMT phenotype with increased migratory and invasive phenotype. The acquisition of EMT phenotype was mediated

via the activation of IGF1R/NF-κB signaling pathway. Inhibition of IGF1R/NF-κB signaling restored the sensitivity to gefitinib and suppressed the migration and invasion capability, suggesting that this pathway could be a novel target for gefitinib-resistant EGFR-mutant NSCLC therapy [38].

To summarize, despite the wide variety of cancer drugs available to treat lung cancer, acquired resistance to therapy is still a frequently encountered problem. It is important to identify the resistance conferring mechanisms, so that the resistance can be circumvented or reversed with novel anticancer drugs. Although drug-resistant cell lines provide robust preclinical models to interrogate underlying mechanisms of resistance and anticancer drug screening, they lack key features present *in vivo* in primary tumors that may provide additional mechanism associated with chemoresistance.

2.2. 3D systems

Although a plethora of 3D systems have been developed for anticancer drug screening at present, despite some technical traits, none of these systems showed a clear advantage over other regarding the ability to select successful clinically useful anticancer drugs. For space limitations, only floating spheroids and floating tumorspheres will be addressed in this chapter. **Figure 1** shows representative microscopy image of spheroids and tumorspheres.

2.2.1. Floating spheroids

The 3D multicellular aggregates formed from single cell suspensions in FBS containing media under anchorage-dependent or independent conditions are commonly known as "Spheroids." It is an excellent 3D model for drug screening as it can closely mimic tumor heterogeneity, tumor microenvironment niches (normoxic, hypoxic, pH gradient zones) and tumor structural as well as functional intricacies. Spheroids with constant size and consistent structural features are necessary to generate consistent and reproducible results.

Researchers have developed various techniques to develop spheroids to achieve these targets. The commonly used 3D cell culture methods can be divided into two main categories—(i) scaffold
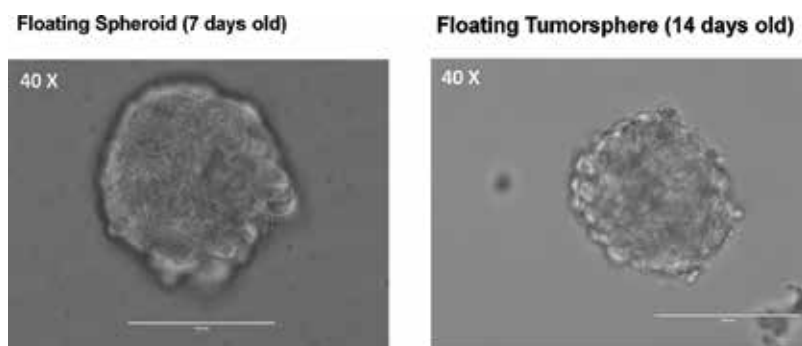

Figure 1. Representative images of H460 lung cancer cells growing as floating spheroids in serum-containing media (A) or as floating tumorspheres in serum-free media (B). Image in B was reprinted with permission from [39]. Bar = 100 μm.

based (hydrogel and inserts) and (ii) non-scaffold based. Scaffold-based methods involve collagen-, chitosan- and polycaprolactone-based biomaterials, which serve as extracellular matrix and provide a 3D architecture for the growth of cells in anchorage-dependent manner in 3D cellular entities. Non-scaffold-based approaches employ techniques such as forced floating, rotary devices, hanging drop arrays, microfluidics, etc. to generate spheroids. Forced floating is the simplest and most commonly used method for the spheroids in a laboratory setting. As the name suggests, forced floating of cells is achieved by culturing them in vessels that have been coated with poly-HEMA or agarose suspensions, which prevents attachment of cells to the substratum. This method has been successfully used to generate spheroids of various types of cancers. It is a simple, inexpensive and convenient method for generating spheroids; however, it produces spheroids of variable sizes and shapes. In a recent study, Ivascu et al. reported the use of centrifugation to generate spheroids of fixed size using forced suspension method [40]. They added cell suspensions containing a fixed number of cells in each well of poly-HEMA-coated 96-well plates and subsequently centrifuged the plates to colocalize them. They reported formation of uniform single spheroids per well as early as 24 hours. Rotary 3D culture techniques work on agitation principle, which prevents cells from attaching to the container and instead cells interact with each other and develop 3D spheroids. Rotary culture methods can be sub divided into two main categories: (i) spinner flask and (ii) rotational culture systems. In spinner flask method, cell suspensions are constantly agitated by magnetic stirrer, while in rotational culture system cell suspension media is moved by rotating the culture vessel itself. Both these methods can produce large amount of spheroids and can be used for long-term production of spheroids. As culture is constant, agitated cells receive constant supply of nutrients and oxygen. However, size and structure of the spheroids cannot be controlled in these 3D culture methods. As these methods rely on constant moving of culture media, it exerts extensive force on cells which can either damage or affect cellular physiology of the cells. Rotational culture system is a superior technique than spinner flask method as it exerts far less force on the cells [41]. Kelm et al. developed hanging drop method for the generation of multicellular spheroids [42]. This method employs gravity as a force to bring the cells together to generate spheroids. Small aliquots of cell suspension media (usually 20 μL) carrying fixed number of cells are placed in wells of microtiter plate. These plates are then inverted and suspension media forms a drop and cells migrate to the tip of the drop due to gravity. These cells are then allowed to proliferate and generate a single spheroid per well. This method is good for high throughput, and spheroids with consistent size can be generated with excellent reproducibility. One limitation of this method is the volume of the liquid (up to 50 μL) that can be used to generate drop as surface tension can keep only small volumes of media together. Since small volumes of media are used for drop generation, this method requires constant media replacement making maintenance of culture challenging [43]. Tan et al. used a microfluidic platform to generate spheres [44]. This platform consists of a main channel and an array of coated microwells. Media containing single cell suspension flows through the channel and the cells get trapped into the wells where they grow in close contact and generate a single spheroid per well. Size and shape of the spheroids can be controlled by this method. It is a very good platform for high throughput drug screening and compatible with multidimensional imaging. However, spheroids generated in microfluidic platform cannot be retrieved for further study and structural analysis. In addition, several coculture methods have been developed to mimic complex cancer cell and microenvironment interactions. Tumor microenvironment is a complex

system that involves interactions between tumor cells and adjacent stroma cells (fibroblasts, endothelial and inflammatory cells) embedded in extracellular matrix (ECM). Spheroids generation using a coculture model by coculturing cancer cells and fibroblasts and/or immune cells has been reported and studied for their unique interaction and subsequent effect on carcinogenesis [45, 46]. Spheroids perfectly mimic an avascular tumor microenvironment and cellular heterogeneity. Similar to tumor a nutrient, oxygen and pH gradient exist in spheroids leading to three distinct cellular zones in the spheroids [47]. The central necrotic zone, which mostly constituted by dead cells, is devoid of oxygen and nutrients. This is followed by middle dormant (senescent) zone consisted of quiescent cells. The peripheral layer, which has sufficient supply of nutrients and oxygen, has proliferative cells. Microelectrodes and proton magnetic resonance with pH-sensitive indicators are commonly used techniques to study oxygen flow and pH inside the spheroids [48, 49]. **Figure 2** illustrates the complex 3 architecture of spheroids.

In general, 3D spheroids show less chemosensitivity towards various drugs compared to 2D *in vitro* cell culture models [50, 51]. This differential response can be attributed to structural and functional complexity of 3D model vs. a 2D model. Drug penetration is one of the reasons of this resistance as the structural and microenvironmental barriers prevent effective convection of drug in 3D spheroids. As a result, drug fails to achieve an effective concentration inside the tumor to deliver a strong anticancer response. Kerr et al. demonstrated that reduced penetration of anthracycline-based drugs was responsible for a mild cytotoxic effect of these drugs in lung spheroids and a more lipophilic analogue partitioned better hence can be a better therapeutic option against 3D spheroids [52]. A recent study by Gupta et al. showed improved efficacy of paclitaxel when coadministered with tumor penetrating peptide iRGD due to increased availability of the drug in the interior of A549 spheroids [53]. Similarly, tumor
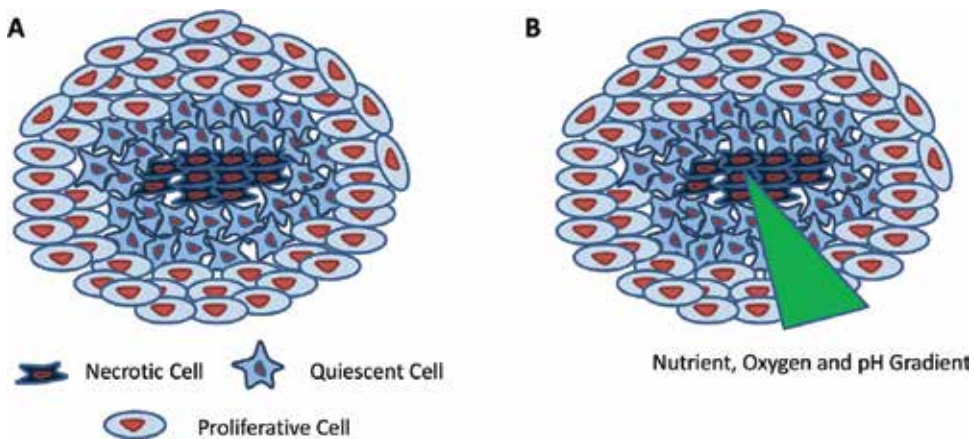

Figure 2. Structural organization of spheroids. (A) Cells are organized in three distinct zones based on viability and proliferative status of the contributing cells. The innermost necrotic layer is mostly composed of dead cells, which is followed by middle senescent layer consisting of slowly growing quiescent cells. The outermost layer is proliferative and contains rapid dividing cells. (B) A gradient of nutrients, oxygen and pH is responsible for this structural organization of cells in spheroids. Since the interior section of the spheroids receives least amount of nutrients and oxygen, the survival of cells in this part of the spheroid is compromised. As supply of nutrients and oxygen improve from moderate to high in the middle and outer section of spheroids, cells change from quiescent to highly proliferative in these zones.

heterogeneity arising from the distinct arrangement of cells in different proliferative zones in spheroids can promote resistance to certain drugs. For example, antiproliferative drugs such as paclitaxel, which confers most activity towards rapidly dividing cells, showed a reduced activity towards interior region of spheroid as it consists of quiescent cells [54]. Drugs like doxorubicin which induce anticancer effect by producing reactive oxygen species show limited response under hypoxic conditions [55]. However, drugs which get activated under hypoxic conditions can potentially treat hypoxic tumors. In 2012, Meng et al. observed that a hypoxia-selective drug TH-302 showed 650-fold greater activity in hypoxic H460 lung cancer cell spheroids than in monolayer cells [56]. Cancer cells switch their metabolism to glycolysis and produce lactic acid in the process. A lack of effective clearing of this excess lactic acid results in accumulation of lactic acid and subsequent reduction of pH in the interior of the tumor. A reduced pH of the tumor microenvironment can adversely affect the cytotoxicity of weak basic drugs such as doxorubicin, mitoxantrone, vincristine, etc. These drugs get protonated under acidic conditions resulting in decreased cellular uptake, hence lose their activity [57]. Activations of mechanisms related to drug efflux in spheroid can be responsible for increased resistance to the drugs. In 2015, Rodriguez et al. showed an increased expression of MDR-1 and P-glycoprotein in spheroids of INER-37 human NSCLC cell line compared to 2D culture likely responsible for increased drug resistance [58]. Several studies have indicated CSC enrichment in spheroids as possible explanation for drug resistance and metastasis [59, 60]. A 3D spheroid platform is a more relevant model for anti-cancer drug screening, and represents a much better approach to achieving therapeutic outcomes for cancer patients as compared to current therapeutic practices. There is need to develop drugs with better penetrating ability to improve its availability to the most interior sections of tumors. Improved drug delivery techniques employing vehicles, such as nanoparticles, liposomes, nanospheres, etc., can (i) improve drug delivery and (ii) protect drug decomposition under harsh tumor microenvironment. Tumor heterogeneity arising from cells of different metabolic, proliferative, chemosensitivity, metastatic and stemness profile calls for a more complex and versatile therapy approach for cancer treatment. For example—a cotherapy approach using multiple drugs which can target different populations of cancer cells will have better outcome for patients than a simple single therapy approach. Development of better drugs with single drugs targeting multiple pathways can remove the need to use multiple drugs simultaneously. So, an effective and improved drug development, drug delivery and drug testing programme are the need of hour to make significant inroads in a fight against cancer. It is important to mention that Steadman et al. raised concerns about the possibility that the chemoresistance mechanisms found in spheroids may differ from the resistance found in intractable solid tumors in patients [61]. If this is the case, drugs selected by this system will be ineffective in primary tumors. Future studies should be aimed to attempt to recapitulate *in vitro,* the complex architecture of lung tumors should take in consideration several factors known to simultaneously contribute to chemoresistance.

2.2.2. Floating tumorspheres

Cancer is a complex disease with several preventive mechanisms put in place to escape elimination by immune response, drug efflux or expulsion mechanisms to mitigate drug activity and a microenvironment uniquely suitable for cancer cells. The matter is further complicated by tumor

heterogeneity resulting in mixed population of cells with varying degree of chemoresistance, invasive and migratory potential. Several cancer models have been proposed to explain tumor heterogeneity and related tumor characteristics. Clonal evolution model (CEM) and cancer stem cell model (CSCM) are two of the most popular and widely accepted models. CEM postulates clonal evolution of tumor and suggests existence of several clones with varying genetic and epigenetic modifications as the contributor for tumor heterogeneity. Lately, CSCM has become most commonly used model for tumor biology. According to CSCM, a rare but fixed population of cells known as cancer stem cells (CSCs) with indefinite self-renewal potential and pluripotency are responsible for tumor origin, heterogeneity, metastasis, resistance and relapse. CSCs are rare cells and most of the times only constitute 1% of tumor volume. Over the period, various methods and techniques have been developed to enrich and study the CSC. Fluorescence-activated cell sorting (FACS) using surface markers, culture of cells in suspension for generating tumorspheres with increased stemness, sorting of cells based on the activity of intracellular enzymes such as aldehyde dehydrogenase (ALDH) and 26S proteosome and sorting of side population cells due to their ability to exclude Hoechst 33342 are some of the most commonly used methods for CSC sorting and subsequent enrichment. Generation of tumorspheres is most convenient and cost-effective way for enriching CSCs as it does not require previous knowledge of surface marker or enzyme expression and costly cell sorting FACS set up. For this chapter, we will focus on generation of floating tumorspheres and their use as a model for studying chemo-resistance. In 1996, Reynolds et al. described the generation of normal neural stem cells as "neurospheres" upon culture of brain cells in floating conditions in serum-free medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF) [62]. Following the same culture protocol, Singh et al. generated tumorspheres from the brain tumor cells [63]. Soon after, the method was widely accepted and researcher all over the world used this protocol to generate tumorspheres from a wide variety of cancers [64–68]. Over the course of time, researchers have tried to improve upon the existing protocol of sphere formation in order to make it more efficient, consistent, reliable and physiologically relevant. Various types of scaffolds consisting of chitosan-alginate, collagen, alginate and agarose have been used as 3D matrices to substitute low attachment plates [69–72]. In 2014, Cao et al. generated tumorspheres from primary neuroblastoma cells driven from MYCN transgenic mice using a medium supplemented with fetal bovine serum (FBS) and β-mercaptoethanol. These spheres exhibited indefinite renewal as they could be passaged more than 20 times and also demonstrated enhanced metastatic potential [73]. More recently, in one of our studies, we described culture of lung tumorspheres (LTs) from H460 cells solely in serum-free media without supplementation of growth factors [39]. This method was further extended to mammospheres (MSs) generation in MCF-7 cells [74].

Over a period, several targeted therapies have been developed towards various oncogenic drivers (EGFR, ALK, ROS1, RET, etc.) in lung cancer [75]. However, most of these drugs have shown a transient effect on cancer as initial remission of disease is followed by outbreak of more aggressive and resistant cancer resulting in modest overall survival. Therapy resistance is a leading hurdle in cancer treatment and mostly responsible for poor outcome for the patients. Several researchers have identified acquired mutations during the prolonged treatment with a single drug as the leading cause for the therapy resistance. As discussed in more detail in the

previous section (Section 2.1.3.), secondary mutations, bypass pathways such as MET, amplification of HER2, overexpression of AXL kinase, etc. have been shown to induce therapy resistance in lung cancer [75–78]. With the advent of concept of CSCs and subsequent discovery of CSCs in several types of cancers, researchers started to explore a possible link between therapy resistance and possible changes in the CSCs population. In 2008, Levina et al. were the first researchers to report the enrichment of cancer stem-like cells (CSLCs) in response to therapy treatment in lung tumor. They observed that the surviving cancer cell expressed stemness-related markers such as CD133, CD117, SSEA-3, TRA1-81, Oct4 and nuclear β-catenin. These cells retained a higher capability to form spheres, self-renewal, differentiation and showed high metastatic and tumorigenic potential [79]. Several studies have pointed towards enrichment of CSLCs as a contributing factor for acquired resistance in lung cancer in response to Cisplatin therapy [25, 80]. Similar findings have been reported for other commonly used anticancer therapies in lung cancer [81]. Normally, a 2D *in vitro* model is used for the screening of potential anticancer agents and more often they fail to translate *in vitro* antiproliferative efficacy of a drug in *in vivo* settings. This is due to an inherently flawed 2D model which does not replicate a real tumor at structural, physico-chemical, mechanical and biochemical levels. A tumor is a 3D entity composed of cells exhibiting varying degree of resistance, proliferative and metastatic tendencies. Tumor microenvironmental conditions which play a critical role in determining tumor heterogeneity, resistance and metastasis cannot be replicated by an *in vitro* model, and hence it often fails to impress upon aforementioned attributes of tumor. Therefore, a more physiologically relevant drug screening tool is a real necessity to improve upon often failing *in vitro* cytotoxicity model. Tumorspheres with their 3D structure and often increased stemness can serve as more resistant and invasive model with closely relatable microenvironmental conditions as that of a real tumor. It can serve as a more realistic approach for drug screening with better chances of replication of drug efficacy in *in vivo* system. Tumorspheres assay can serve as a quick and more economical intermediary testing platform for *in vivo* tumor xenograft studies in a high-throughput setting while *in vivo* studies can be reserved for validating findings observed in the tumorsphere assays.

In recent years, researchers have successfully employed tumorsphere model for effective screening of potential anticancer drugs. A number of studies exploring anticancer efficacy of different classes of compounds such as inorganic [82], natural ingredients [83], antibiotics [84], Chinese medicine [85], cardiac glycosides [86], etc. against LTs have been published. *In vivo* studies have been performed to explore and extend observed drug efficacy against LTs in a more physiological setting [87, 88]. Several studies have tried combination therapy approaches in LTs setting to develop effective drug combinations to alleviate therapy resistance-related concerns and improve efficacy of existing cancer drugs [68, 89–92]. The promising anticancer drugs selected using tumorspheres described above need to pass the test of more relevant animal models and later on successful clinical trials to validate the applicability of this 3D system for anticancer drug screening.

2.2.3. 3D systems as models to test drug delivery and efficacy

Drug delivery refers to approaches, formulations, technologies and systems for transporting and administering *in vivo* an active pharmaceutical ingredient (API) to achieve a therapeutic effect in

the patient. In tumors, there are gradients of drug concentrations, oxygen and nutrients created by the distance from blood vessels. These similarities make 3D systems the more physiological models for drug delivery testing and therefore better predictors of chemosensitivity. Metha et al. [93] described in detail six characteristics of spheroids, which are absent in conventional culture formats, that mimic how drug delivery might occur *in vivo*: (1) spheroids model the 3D architecture of tissues, including multicellular arrangement and extracellular matrix deposition, found *in vivo*. (2) Spheroids have sizeable cell-cell interactions, including tight junctions that are known to influence response of cells to drugs, (3) spheroids have diffusional limits to mass transport of drugs, nutrients and other factors, (4) spheroids are formed with two or more cell types in varying ratios representing intercellular signaling and architecture that can help to understand how multiple cell types might impact drug delivery, (5) rare cells such as cancer stem cells or primary stem cells may be present or incorporated and maintained in spheroids which can facilitate targeting these specific cells with drugs and (6) larger spheroids develop central necrosis and regions of hypoxia present in many cancers. These specific microenvironments have been shown to contain cancer cells with increased chemoresistance. Tumorspheres share most of these characteristics with few differences: (1) they are considered to be enriched with CS-LCs that are inherently more resistant than non-CS-LCs and (2) tumorspheres can be generated in complete absence of external mitogenic stimulation that makes ideal system to study how specific factors (sometimes present in serum-containing media) may alter the response of cancer cells to anticancer drugs. For instance, lung tumorspheres grown in the absence of external mitogenic stimuli when exposed to exogenously added EGF demonstrated increased sensitivity to Erlotinib and Gefitinib [10]. In summary, despite their limitations, both spheroids and tumorspheres are useful and complementary systems for drug delivery testing.

3. Implications for translational oncology

The *in vitro* identification of effective anticancer is a crucial part of the anticancer drug screening program. Drug development is a long and expensive business, and billions of dollars are invested for a single successful drug release in market. It starts at identification of potential drug candidate with subsequent testing *in vitro* and *in vivo* setting followed by testing in various phases of clinical trials. Upon successful completion of clinical trial, drug is approved by FDA for marketing. Therefore, development of realistic models of drug screening is extremely important for vetting of drug candidates in earlier preclinical stages for them to have better chances to be successful in later clinical trials. Traditionally, 2D *in vitro* culture model is used for cancer drug screening. However, it completely fails to recapture finer intricacies of 3D tumor. It does not have any semblance with a 3D tumor at microenvironmental, biological and physiological levels and hence in most instances miserably fails in therapy translation from an *in vitro* to an *in vivo* setting. In order to address these issues, researchers have developed various drug screening 3D models, which align well, mimic essence of natural tumors and carry more significance as drug screening platforms. Despite these important technical advances, few drugs have been translated into clinical practice and the prognosis of lung cancer patients remains poor, suggesting that current *in vitro* 3D models are still not good models of primary tumors.

4. Conclusions

At present, current *in vitro* 3D models offer significant advantages over 2D systems in terms of recapitulating intratumoral heterogeneity and enrichment of cancer stem cells and have been extremely useful for understanding cancer cell biology. However, for anticancer drugs discovery, this success has not been translated into the clinic because the prognosis of lung cancer patients still remains poor. Future development in the field should concentrate on (i) efforts to better mimic *in vivo* conditions and (ii) identifying the underlying mechanism of chemoresistance of *in vitro* system in correlation with *in vivo* conditions. Otherwise, drugs selected with the current method will only target a subpopulation of chemoresistant cells or as suggested by Steadman et al. will be ineffective due to differences in the underlying mechanism of chemoresistance between *in vitro* 3D system and *in vivo* conditions.

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Conflict of interest

The author declares no conflict of interest, financial or otherwise.

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Among the deadliest type of cancers, lung cancer faces several challenges in diagnosis and treatment: late diagnosis and misdiagnosis, inadequate tumor sampling, and resistance development to current therapies, among others. Together with advances in the understanding of molecular features, factors, and mechanisms involved in initiation and tumor progression, important improvements have occurred in diagnostics and therapeutics in the shape of advances in molecular genotyping, procedures for sampling, new potential, and less invasive sources of samples for the diagnosis and development of new targeted therapies. The aim of this book is to provide an exciting read on strategies in the diagnosis and therapy of lung cancer.

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